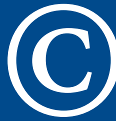

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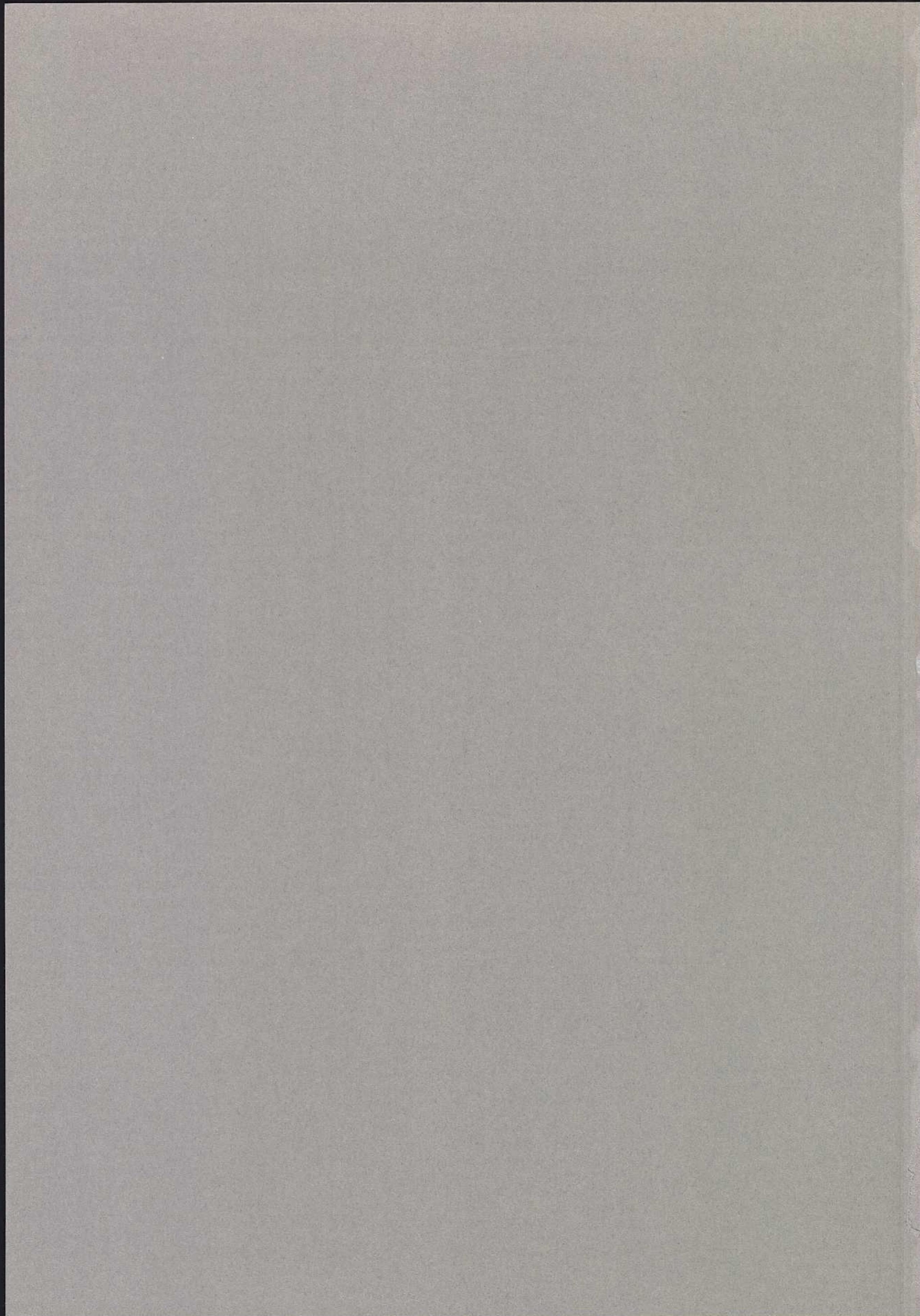
1761

REGULATION OF ANTI-THROMBOGENETIC FACTORS IN ENDOTHELIUM

prostacyclin, tissue plasminogen activator and plasminogen activator inhibitor type-1

Hans E Rydholm

Göteborg 1997



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AKADEMISK AVHANDLING

som för avläggande av medicine doktors examen
vid Göteborgs Universitet
kommer att offentligen försvaras
i aulan, Centralkliniken, Östra Sjukhuset,
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av

HANS E RYDHOLM
leg. läkare

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I. Rydholm H, Boström S, Hansson G, Risberg B
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Thrombin signal transduction of the fibrinolytic system in human adult venous endothelium.
Submitted for publication.

Abstract

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Hans E Rydholm, Department of Surgery, Sahlgrenska University Hospital, Östra, Göteborg, Sweden.

Endothelial cells, producing both pro and anti-thrombogenetic substances are central to the cardiovascular system. A basic knowledge of the pathway which regulates the synthesis of the anti-thrombogenetic substances, prostacyclin (PGI_2), tissue plasminogen activator (t-PA) and the fast inhibitor, plasminogen activator inhibitor type-1 (PAI-1), is crucial in order to modulate a dysfunctional endothelium in cardiovascular diseases.

The aim of the present investigation was to clarify important aspects of the regulation of anti-thrombogetic factors in endothelial cells of different origin, in particular the influence of adrenergic control in PGI_2 production and the regulation t-PA and PAI-1 production.

Endothelial cells from human umbilical and adult great saphenous veins were harvested and cultured. The production of PGI_2, t-PA and PAI-1 antigens were analyzed from the conditioned medium. The regulation of the cellular synthesis was studied by adding defined agonists or antagonists to receptors or intracellular transduction pathways.

Adrenergic stimulation with isoprenalin increased PGI_2 production from endothelial cells. The β-adrenoceptor blocker, propranolol or metoprolol, increased the effect of isoprenalin, but had no effect per se. Different isomers of metoprolol with a high or low receptor blocking effect produced equal results.

In dose-response studies, the production of t-PA and PAI-1 from umbilical and adult endothelial cells was increased by phorbolester or thrombin. At high concentrations, endotoxin also increased the production from umbilical but not from adult cells. Isoprenalin, ephedrine or endothelin-1 had no influence. Forskolin decreased the production of PAI-1, and Na-nitroprusside increased it slightly. Neither of these substances influenced t-PA production.

In "cross-talking" studies, the production of t-PA and PAI-1 could be modulated by co-incubating different substances. The protein kinase C inhibitor H7 decreased the t-PA and PAI-1 production augmented by endotoxin, thrombin and phorbolester, indicating an on-going signal transduction. Combinations with forskolin generally decreased agonist-induced PAI-1 production. The effect on t-PA production diverged between umbilical and adult cells when endotoxin, thrombin or phorbolester were co-incubated with forskolin.

In transduction studies, the thrombin-induced t-PA and PAI-1 production from adult endothelial cells was decreased by pertussis toxin or genistein, representing $G_{\alpha i}$-protein and tyrosine kinase pathway inhibitors respectively.

In conclusion: The production of prostacyclin from endothelial cells is under adrenergic control, modulating the effect of β-adrenoceptor blockers. Signal transduction through adenylate cyclase, protein kinase C and tyrosine kinase pathways is critical in the regulation of t-PA and PAI-1 production from endothelial cells. Interference with these systems can be one way of modulating the balance between coagulation and fibrinolysis in the blood vessel. Endothelial cells of different origin regulate the production of t-PA and PAI-1 differently.

Key words: endothelium, epoprostenol, b-adrenoceptor, plasminogen activator, plasminogen activator inhibitor, signal transduction, tyrosine kinase, GTP-binding protein, cell origin.

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REGULATION OF ANTI-THROMBOGENETIC FACTORS IN ENDOTHELIUM

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Hans E Rydholm

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Contents

	page.
Abstract	ii
List of publications	v
Abbreviations	vi
INTRODUCTION	1
Endothelial interactions	1
Platelets	2
Leukocytes	2
Erytrocytes	2
Flow-Sheer stress	3
Smooth muscle cells	3
Extra cellular matrix	3
Prostacyclin (PGI2)	4
PLA2 - AA	4
Agonist-regulated production	4
PGI2, hypertension, adrenergic tonus	4
Coagulation	5
Blood clot	5
Coagulation cascade	5
Endothelium - coagulation	6
Platelets - coagulation	6
Fibrinolysis	6
General	6
Plasmin	7
a2-antiplasmin	7
Plasminogen activatior	7
Plasminogen activator inhibitor	7
The fibrinolytic system	8
Tissue plasminogen activator (t-PA)	8
Release - Regulation	9
Urokinase (u-PA)	9
Other fibrinolytic activators	10
Plasminogen activator type-1	10
Active - latent -vitronectin	11
Release - Regulation	11
Plasminogen activator type-2	12
Other plasminogen activator inhibitor	12
Interactions between PA`s - PAI`s	13
Fibrin interactions	13
Complex formation - Clearance	13
Signal transduction	14
Main transduction systems	14
GTP-binding system	14
Adenylate cyclase	15
Phospholiphase C/D -Protein kinase C	15
Mitogen activated protein kinase (MAP) system	16
General intracellular signal systems	16
Ca2+ - system	16
Transcription factors	17
Intracellular effector systems	18
Fast enzyme system, AA, PGI2	18
Slow gene-transcription systems, t-PA, PAI-1	19
AIMS of the study	20
MATERIALS and METHODS	21
Drugs	21
Cell culture	22
Biochemical analysis	22
Prostacyclin	22
t-PA, PAI-1	22
Statistical methods	23

Experimental design 23
RESULTS 24
Prostacyclin 24
Basal production 24
Stimulated production 24
Time-course 26
t-PA / PAI-1 26
Basal production 26
Agonist stimulated production 26
HUVEC 26
HAVEC 27
Modulated production "Cross-talk" 28
Co-incubation with H7 28
Co-incubation with forskolin 28
Thrombin signal transduction 30
Pertussis toxin 30
Genistein, Vanadate 31
DISCUSSION 32
Discussion of methods 32
Prostacyclin 32
t-PA / PAI-1 32
Prostacyclin 33
PGI2, hypertension, adrenergic tonus 33
Results 33
Signal transduction - Hypothesis 34
Interactions with PLA2 35
Ca2+ 35
MAP kinase 35
Interactions with the beta adrenoceptor 36
Interactions with other pharmacological side effect 36
Summary 37
t-PA / PAI-1 37
Isoprenalin / Ephedrine 38
Forskolin 38
Na-nitroprusside 38
Endothelin-1 39
Phorbol ester / protein kinase C / H7 39
Endotoxin 40
Thrombin 41
Pertussis toxin 42
Genistein / Vanadate 42
Hypothesized signal transduction in HAVEC 43
Endothelial heterogeneity 44
Endothelial heterogeneity 44
Qualitative differences 45
Intracellular origin hetrogeneity 45
External dynamic hetrogeneity 46
Quantitative differences 46
Summary 46
SUMMARY 47
CONCLUSIONS 48
ACKNOWLEDGMENTS 49
REFERENCES 50 - 62
PAPERS I- IV

Lists of publications

This thesis is based on the following publications and manuscripts, which are referred to in the text by their Roman numerals:

I. Rydholm H, Boström S, Hansson G, Risberg B
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Submitted for publication

Abbreviations

AA	Arachidonic acid
AC	Adenylate cyclase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Activated protein C
ATF	Activating transcription factor
bFGF	Basic fibroblast growth factor
cAMP	cyclic AMP
cGMP	cyclic GMP
COX	Cyclooxygenase
CRE	cAMP response element
CREB	CRE binding protein
CV	Coefficient of variation
DAG	Diacyl glycerol
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
ECGF	Endothelial cell growth factor
ECM	Extra cellular matrix
ET	Endothelin
FV-VIII	Coagulation factors (V-XIII)
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HAVEC	Human adult EC
HUVEC	Human umbilical EC
H7	Isoquinolinyl piperazine
IL-(1-8)	Interleukin- (1-8)
IP3	Inositol trisphosphate
LPS	Lipopolysaccaride
MAP	Mitogen activated protein
Mr	Relative molecular mass
NFkB	Nuclear factor kappa beta
NO	Nitric oxide
PA	Plasminogen activator
PAF	Platelet activating factor
PAI-1	PA inhibitor type-1
PAI-2	PA inhibitor type-2
PGI_2	Prostacyclin
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
Plg	Plasminogen
PMA	Phorbolester
PTX	Pertussis toxin
SMC	Smooth muscle cell
t-PA	Tissue plasminogen activator
sct-PA	Single chain t-PA
scu-PA	Single chain u-PA
tct-PA	Two chain t-PA
tcu-PA	Two chain u-PA
TF	Tissue factor
TF´s	Transcription factor
TGFβ	Transforming growth factor β
u-PA	Urokinase (PA)
uPAR	Urokinase receptor
vWF	von Willebrand factor

INTRODUCTION

The primary function of the cardiovascular system is to distribute and exchange dissolved substances and mobile cells between different parts of the body. At the same time, the system must be able to react to local trauma, protecting the individual from life-threatening bleeding by closing parts of the system. These contradictory demand have created many interacting control systems regulating the fluidity of the blood. Unfavourable shifts in this balance will appear as cardiovascular disturbances, which is an important causes of morbidity and mortality.

Endothelial cells (EC), with their unique position between the blood and the tissue, and their high production of regulatory substances, are central to the cardiovascular system. In the normal, uninjured state, the vascular surface is anti-thrombotic, but it could become pro-thrombotic if pertuburated. This anti-thrombotic function of EC is based on the production of nitric oxide (NO= endothelium-derived relaxing factor, EDRF) (Moncada 1990), prostacyclin PGI_2, (Levin 1984), tissue plasminogen activator (Kooistra 1994), thrombomodulin (Dittman 1995) and heparin-like glycosaminoglycans (Lindblom 1991). Several other factors produced and expressed by EC could act either pro- or anti-thrombotically (Børsum 1991, Risberg 1996). Many complex feedback mechanisms exist, adjusting local vasotonus and platelet aggregation (Vane 1990, Lüscher 1990, Marcus 1993). Basic knowledge of the pathways which regulate the synthesis of thrombogenetic and non-thrombogenetic substances is crucial when trying to modulate the dysfunctional endothelium in cardiovascular diseases. This thesis focuses on the endothelial synthesis of the non-thrombogenetic substances: prostacyclin (PGI_2), tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1).

The final release of substances from endothelial cells is the integrated sum of multiple intra- and extra-cellular interactions. Regulating signals come to the cell surface from both substances in plasma blood-borne cells and also from the tissue beneath the cell. Several signal transduction pathways could interact intracellularly, modulating the external signals before they reach the DNA promotor (Liscovitch 1992, Walsh 1994, Hill 1995, Post 1996). This rather complex picture requires an overview of: 1) endothelial interactions with other cells and plasma, 2) prostacyclin, extracellular influence, 3) coagulation, 4) fibrinolysis, extracellular influence, 5) intracellular signal transduction.

Endothelial interactions

The endothelial cell has an unique position between the blood and the tissue interacting with several different types of cells and soluble substances. Signals from platelets, leukocytes, erythrocytes and flow on the apical side and extracellular matrix and smooth muscle cells on the basal side are integrated. The principles of these interactions are reviewed in the following section.

Platelets

Platelets, being the first to react in the primary hemostasis could interact with the endothelial cells in a variety of ways. The sequence leading to a platelet thrombi will be reviewed in the coagulation section.

The uninjured endothelium close to the aggregating platelet could regulate the spreading of the thrombi by producing prostacyclin (PGI_2), 13-HODE, nitric oxide (NO) and expressing ADPase activity. PGI_2 binds an adenylate cyclase-linked

platelets receptor, decreasing intracellular Ca^{++}, phospholipase C (PLC), and protein kinase C (PKC) activity. NO diffusing through the platelet membrane increases cyclic GMP (cGMP). PGI_2 and NO could act synergistically, decreasing thromboxane production, cytoskeleton contraction and the occurrence of GPIIb/IIIa on the cellsurface. ADPases on the surface of endothelial cells metabolizes pro-aggregating ADP to anti-aggregating adenosin (Kroll 1989, Marcus 1993).

On the other hand, pertuburated endothelium could produce the platelet-aggregating factor (PAF), vWF and GPIIb/IIIa integrin, increasing platelet adhesion and aggregation (Wu 1995, Risberg 1996).

Leukocytes

The specific function of the leukocytes is to recognize and react to "foreign" substances. The endothelium is an active "middleman", transducting messages from the tissue to the leukocytes and guiding the immune-competent blood cells into the affected tissue.

Leukocytes are first captured to "roll" on the endothelial surface by a family of adhesion molecules named selectines. By further interaction between integrins on activated leukocytes and cytokin-induced adhesion molecules (ICAMs) on endothelium, the leukocytes become firmly attached (Bevilacqua 1993, Tedder 1995). Adhered neutrophils could degranulate at the endothelial surface, creating severe injuries, or migrate towards a chemo-attractant stimulus through the endothelial line into the tissue (Bratt 1995).

Endothelial cells harbour P-selectins in Weibel-Palade granule and could express this transient selectin only minutes after being stimulated by thrombin or leukotrienes. Simultaneously-produced PAF activates leukocyte integrins. Cytokines (IL-1, TNFα, IL-8) from activated tissue or blood macrophages bind to specific receptors on the endothelial cells. On being thus stimulated, the endothelium, after a lag-phase, will express the more long-lasting E-selectin and several types of immunoglobulin adhesion molecules (ICAM, VCAM) that could interact with specific counterparts on blood leukocytes (Collins 1995). Endothelial cells could also down-regulate the effects of adhesion molecules and cytokins by producing inactivating soluble receptors that could bind to and inactivate their counterparts (Bonfari 1989, Tedder 1995). Endothelial cells could also act as antigen-presenting cells interacting with T-helper cells to activate B and killer cells (Libby 1991).

Erythrocytes

Red blood cells incorporated in an early thrombi have been demonstrated to be prothrombotic, probably by interfering with adenosin and the NO metabolism (Marcus 1993).

Flow - Sheer stress

Sheer stress could modulate several endothelial cell functions and structure by influencing GTP-binding proteins (Berthiaume 1992), intracellular Ca^{++}, K^+ (Olsen 1988) concentration and tyrosine kinase activity (Berk 1995). Flow could rearrange cellular morphology, increasing prostacyclin (Brunkwall 1988, Berthiaume 1992), nitric oxide (Berk 1995), endothelin (Lücher 1990), t-PA (Diamond 1989) and proteoglycan production (Grimm 1988), thus this will creating a balance between vessel-dilating flow and contracting tension.

Smooth muscle cells (SMC)

Smooth muscle cells (SMC), in their contractile phenotype, are responsible for the vascular tone. Following vascular injuries with collagen net disruption and growth factor exposition, SMC could change into a synthesizing and migrating phenotype (Leeuwen 1996).

Endothelial cells produce several substances that could modify the activity of SMC myosin light chain kinase and contraction by interfering with the SMC intracellular Ca^{++} distribution. Nitric oxide (NO) and prostacyclin (PGI_2) could relax, whereas endothelin (ET) and some cyclooxygenase products (5.6-EET) could contract SMC (Lüsher 1990, 1992, Vane 1990). The endothelially-produced, platelet-derived growth factor (PDGF) and endothelin have a proliferating influence, whereas nitric oxide, prostacyclin, TGFβ and heparan sulphate have a growth-inhibiting influence on SMC (Busse 1993, Fager 1995).

Extra cellular-matrix (ECM)

The extra-cellular matrix (ECM) is a complex dynamic structure interacting with the surrounding cells. In principal, ECM is build of collagen, glycoproteins such as fibronectin, lamelin, vitronectin and heparin-like proteoglycans (Ruoslahti 1985). The glycoproteins could be bonded to specific receptors on the surface of the endothelial cells. These receptors, mainly located to the basal cell surface, could be regulated as regards positioning and timing by the cell. Ligand-receptor interaction further initiates tyrosine kinase activity at focal adhesions (FAK) and signal transduction to the nucleus (Buchanan 1993, Moolenaar 1995, Erpel 1995). The composition of the matrix could influence the endothelial phenotype and the shape and production of substances such as t-PA, PAI-1 and PGI_2, through this pathway (Gillis 1996).

The ECM is also a storage for several cellular-produced and/or interacting substances such as vWF (Wu 1995), bFGF (Hawker 1994), plasminogen (Hajjar 1986), active PAI-1 (Schleef 1990), latent TGFβ and tissue inhibitors of metalloproteinases (TIMP´s) (Magnatti 1993, Leeuwen 1996).

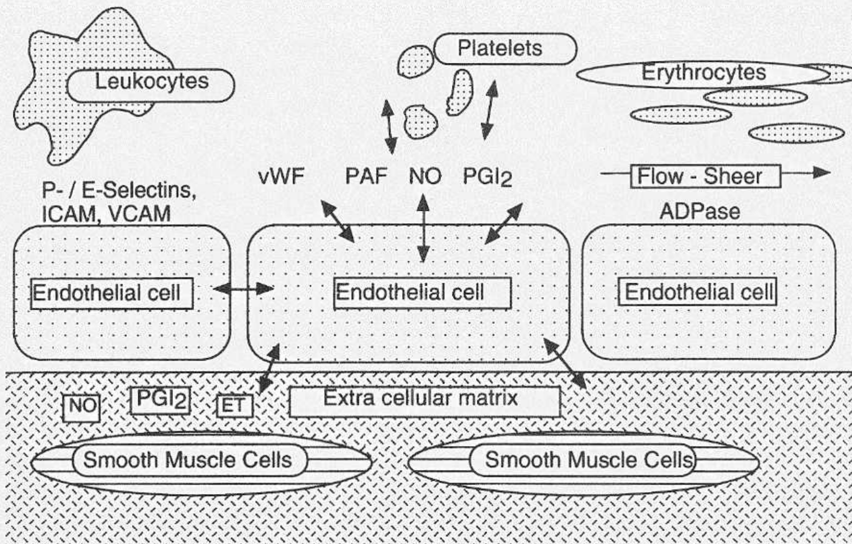


Fig. 1 Endothelial interactions. Summary, corresponding to the text in this section.

Prostacyclin

Endothelial cells (EC), produce high quantities of prostacyclin (PGI_2), an eicosanoid in the prostaglandin family. In contrast to nitric oxide, this lipid metabolite is not produced constitutively but as a result of endothelial stimulation. PGI_2 has a short half-life with effect in the local vessel in synergy with nitric oxide. In the vascular lumen, PGI_2, binding a specific platelet receptor, could decrease the tendency of platelets aggregation and at high concentrations even prevent adhesion. In the vascular wall, PGI_2 relaxes smooth muscle cells (SMC) by interacting with their Ca^{++} flux. Prolonged prostacyclin exposition has even been found to prevent SMC hypertrophy (Levin 1984, Dusting 1990, Lüscher 1990, Vane 1990, Uehara 1991).

Phospholipase A_2 - Arachidonic acid

Phospholipids in the cell membrane are cleaved, preferably by the enzyme phospholipase A_2 (PLA_2), to produce arachidonic acid. This enzyme is important but also potentially destructive for the cell and has to be strictly regulated. The organism, in a complex system with many potential steps to regulate, produces different eicosanoids. Starting from arachidonic acid, different prostaglandins are produced locally in a consecutive series of enzyme reactions according to which tissue-specific enzymes are available (Liscovitch 1992, Dusting 1990, Roberts 1996). (See below).

Agonist-regulated PGI_2 production

The production of PGI_2 from endothelial cells in response to extra-cellular stimuli has been studied extensively. In cell-culture systems, increased synthesis has been reported for: thrombin, histamine (Jaffe 1987, Garcia 1991, Weigel 1991), bradykinin, leukotrienes (Clark 1986), endotoxin, PMA (Nawroth 1984), TNFα, IL-1 (Kawakami 1986), interferon (Eldor 1984), complement (Suttorp 1987), endothelin (Vane 1990, Busse 1993) and shear stress (Frangos 1985). Inhibitory effects have been reported for: salicylic acid, non-steroid anti-inflammatory drugs (NSAID) (Gerritsen 1996), steroids (de Caterina 1986), growth factors (Chung-Welch 1988), hypoxia (Madden 1986), and radiation (Eldor 1987).

Prostacyclin, Hypertension, Adrenergic tonus

A triad of low prostacyclin production, hypertension and high adrenergic tonus has been reported in various studies - in vivo on humans (Beckmann 1988) and rabbits (Åblad 1988), and in vitro on isolated guinea pig hearts (Löbel 1985) and rat aortas (Nishimiya 1990, Hiriwa 1991). These studies supported the hypothesis that decreased prostacyclin production could be a pathophysiological adaptation to the (over-)active adrenergic system and an etiologic factor in hypertension.

The weakest link in this hypothesis is the lack of explanation as to how adrenergic stimuli could influence prostacyclin production from endothelial cells. Some authors have studied this subject using thrombin- or ionofor-stimulated cells (Adler 1981, Brotherton 1982, Hong 1983, Whorton 1985) or chloalose-treated rabbits (Pettersson 1991). The cellular basis for the regulation is not yet completely understood.

In the present study (paper I), using an non-stimulated experimental model, we have tested the hypothesis that prostacyclin production is regulated by adrenergic mechanisms.

Coagulation

The blood clot

The blood clot is a complex structure comprising a fibrin net-structure, adherent platelets and other blood cells. The clot is in a state of permanent turnover creating a dynamic balance with the local environment. Interaction occurs with the fluid and cellular phases of the blood and also with the injured tissue and living cells of the vessel wall (Mosseson 1990, Marcus 1993, Blombäck 1996, Handt 1996).

The coagulation cascade

The fibrin net is the final product in a long series of proteolytic enzymes –the coagulation cascade. In the blood, all the coagulation factors exist in very low concentrations and to be able to react in vivo, they have to be brought together, bound to specific surfaces. Phospholipids in the platelets or endothelial cell membrane could act as binding surfaces.

There are mainly two ways to start the coagulation cascade. These are the "internal" and the "external" pathways. In vivo, the "external" pathway seems to be the most important. The "internal" pathway, starting with the activation of factor XII by collagen or matrix proteins, is probably less powerful (Roberts 1995). The critical step in the initiation of the "external" pathway is the expression of a specific lipoprotein named Tissue Factor (TF). TF is produced by many activated cell types including endothelial cells, macrophages, monocytes, foam cells. TF is also bound to the cellular surface, the subcellular matrix and to the central cores of atherosclerotic plaque. TF can complex-bind coagulation factor VII, the binding de-conformates the molecule and dramatically increases the enzyme activity (Camerer 1995).

The final activator in the coagulation cascade is thrombin, an enzyme with a broad substrate specificity. Thrombin, by cutting off two polypeptides, polymerises fibrinogen into long fibrin filaments. Simultaneously, thrombin activates factor XIII that further degrades and uncovers cross-linking sites on fibrin filaments, completing the fibrin net structure (Mosseson 1990, Blombäck 1996).

Regulatory feedback mechanisms

There are several regulatory feedback systems in the coagulation cascade (Roberts 1995). The formation of fibrin could be inhibited by:

a) antithrombin III (ATIII), a liver-synthesized serine proteinase inhibitor. ATIII, in complex with heparin-like glycosaminoglycans on the surface of endothelial cells, inactivates several coagulation factors including factors Xa, IXa, XIa, XIIa and thrombin.

b) thrombin in complex with endothelial surface-bound thrombomodulin could bring Protein C and S together, creating Activated Protein C (APC). This proteolytic enzyme cleaves and inactivates the coagulation factors Va and VIIIa. APC could also bind and inactivate PAI-1, stimulating fibrinolysis (Krishnamurti 1992, Dittman 1995).

c) the rate-limiting step in the "external" coagulation cascade, the tissue factor in complex with FVII, could be effectively inhibited by activated factor X in complex with the tissue factor pathway inhibitor (TFPI or EPI). Endothelial cells and platelets (megakaryocytes) are the source of TFPI (Camerer 1995, Petersen 1995).

Endothelium-Coagulation

The endothelial cell, being either pro- or anticoagulant, has a central and pivotal role in the dynamic turnover of the blood clot (Risberg 1996).

Procoagulant endothelium

When perturburated the endothelium could initiate coagulation, platelets / leukocyte adhesion and vascular smooth muscle contraction. The procoagulative state is created by the appearance of the tissue factor and adhesion molecules such as integrins (GPIIb/IIIa, vitronectin receptor), selectins (P-, E-selektin), and the Ig-superfamily (ICAM, VCAM) on the cell surface. Thrombosis formation is also facilitated by the endothelial secretion of the von Willebrant factor (vWF), factor VIII, P-selectin, plasminogen activator inhibitor type 1 (PAI-1), platelets activation factor (PAF) and endothelin (ET) (Børsum 1991, Bevilacqua 1993, Buchanan 1993, Wu 1995).

Anticoagulant endothelium

The endothelium could reduce the growth of the fibrin clot, counteracting platelets aggregation / adhesion and promoting vascular smooth muscle relaxation. The anticoagulativ state is created by the expression of heparin-like glycosaminoglycans and thrombomodulin on the cell surface. Further secretion of nitric oxide, prostacyclin, 13-HODE, TFPI and t-PA acts as an anticoagulant (Moncada 1990, Børsum 1991, Buchanan 1993, Kooistra 1994, Petersen 1995, Dittman 1995).

Platelets-Coagulation

Platelets adhere / aggregate and initiate vascular constriction in the primary hemostasis and also by interacting with the coagulation cascade and the endothelium co-operate in the creation of a fibrin-stabilised thrombi. When exposed to the blood in injured tissue, vWF bound into the sub-endothelium could adhere and activate platelets (Wu 1995). The activated platelets release coagulation factors, serotonin, ADP, Ca^{++} and PAI-1. They also start to produce thromboxan. Simultaneously, coagulation binding surfaces and aggregating integrins (GPIIb/IIIa) will be exposed on the platelet surface. Coagulation factors will be bound and active on the platelet surface, producing thrombin and creating a positive feedback loop. Fibrinogen and vWF molecules could bind surface integrins and promote platelets aggregation (Marcus 1993). Platelets-bound PAI-1, although mostly in the latent phase, could protect the thrombi from lysis (Declerch 1988, Handt 1996, Robbie 1996). By expressing P-selectin and secreting chemotactic substances such as 12-HETE, activated platelets could attract neutrophils to be built into the thrombi. Neutrophils have antiplatelet-aggregating and thrombolytic potential but also induce inflammatory reaction by secreting leucotriens (LTB4). Red blood cells incorporated in the fibrin net are prothrombotic probably by interfering with adenosin, NO metabolism (Marcus 1993, Soo 1996).

Fibrinolysis

General schemes

Fibrinolysis has revealed itself to be a central biological mechanism involved not only in vessel wall anti-thrombogenicity, but also in cellular migration processes such as angiogenesis or malignancy (Pepper 1993, Blasi 1993, Mignatti 1993, Leeuwen 1996). The balance between coagulation and fibrinolysis plays a pivotal role in the

regulation of the fibrin clot. Disturbances in blood fibrinolytic parameters have been associated with myocardial infarction and identified as an important prognostic risk factor for several neoplasms (Hamsten 1993, Brünner 1994, Juhan-Vague 1996). Accordingly deep vein thrombosis epidemiological studies have presented diverging results, probably due both to methodological difficulties and the great variety of interacting factors (Eriksson 1991, Takada 1994, Wiman 1995).

Many different types of cells could produce proteins that influence the fibrinolytic balance (Hart 1988, Loskutoff 1989, Kooistra 1990). Owing to their location between the blood and the tissue and their high production of t-PA, u-PA, and PAI-1, endothelial cells are central to vascular fibrinolysis and angiogenesis (v Hinsbergh 1987, Kooistra 1994).

Plasmin

Plasmin, the potent proteolytic enzyme, is the active principal in the fibrinolytic system. Active plasmin degrades not only fibrin but also proteins in the extracellular matrix (Mignatti 1993, v Leeuwen 1996) and cell receptors (Schafer 1987). Plasmin could also activate single-chain t-PA / u-PA, latent TGFβ, latent collagenase, and release bFGF from the matrix (Mignatti 1993, Plow 1995, v Leeuwen 1996). This rather non-selective proteolytic potential has to be strictly controlled by the organism both as regards positioning and timing.

α_2-antiplasmin

With a half-life of about 1 second, free active plasmin is inactivated by proteinas inhibitors. The most important inhibitor has revealed itself to be α_2-antiplasmin while α_2-macroglobulin and α_1-antitrypsin are much less effective (Collen 1991). α_2-antiplasmin is a glycoprotein produced by the liver with a Mr of about 70 000 and a half-life of 2-3 days. This inhibitor, belonging to the serpin family, catches plasmin in a two-step reaction, where the first lysine-binding step could be inhibited by fibrin or 6-aminohexanoic acid (6-AHA) (Collen 1991). α_2-antiplasmin is also incorporated in the fibrin clot, preventing premature plasmin lysis (v Giezen 1993).

Plasminogen activation

Plasminogen (Plg) is a 92 000 Mr glycoprotein with a half-life of about 3 days, synthesized by the liver (Collen 1991). Through the lysine-binding sites in the "kringle" domains, it could be linked to fibrin, cell surfaces or the extracellular matrix (Rånby 1982, Hajjar 1986, Knudsen 1986, Redlitz 1995). Plg is a circulating pro-enzyme that could be cleaved and converted to active plasmin by plasminogen activators (PA). The most important PA´s are tissue plasminogen activator (t-PA) and urokinase (u-PA). Owing to its different characteristics, t-PA seems to be the predominating PA in plasma, and u-PA is the most active PA in the tissue (Rijken 1995). However, results from gene knock-out experiments, have indicated that t-PA and u-PA could be exchangeable (Carmeliet 1995, Lijnen 1996).

Plasminogen activator inhibitors

Plasminogen activator inhibitors (PAI`s) act as specific substrates for t-PA or u-PA, rapidly creating inactive complexes (Hekman 1988, Loskutoff 1989). Several types of PAI exist. Normally, PAI-1 seems to be the most important inhibitor in plasma. The role of PAI-2 is more controversial, probably having more tissue and intracellular functions (Dear 1995, Rijken 1995). Other inhibitors such as α_2-macro-

globulin, C1-inhibitor, PAI-3 (APC inhibitor) and α_2-antiplasmin are much less effective, probably having an effect only during pharmacological thrombolysis (Sprengers 1987, Chandler 1996).

Therapeutically-added streptokinase forms a stable non-inhibitory complex with plasminogen (a half-life of about 25 minutes) and creates large amounts of plasmin (Collen 1991).

The fibrinolytic system

The net fibrinolytic activity is a function of production, complex formation and clearance of PA and PAI-1 respectively. In the small peripheral vessel, the activity is also determined by the local blood flow. The fibrinolytic system is carefully restricted by the regulation of the synthesis, release, activation, localization and inhibition of the participating substances (Chandler 1996).

Gene knock-out experiments have demonstrated that substances in the fibrinolytic cascade could be exchanged. Single knock-out of u-PA or t-PA presented increased thrombus formation only when pertuburated, whereas double knock-out u-PA + t-PA or single knock-out of plasminogen or genes expressing high PAI-1 all demonstrated spontaneous thrombosis. On the other hand, PAI-1 knock-out produced bleeding and the incapacity to form thrombi. Even neo-intima formation could be influenced by gene knock-out showing u-PA important activator and PAI-1 to be an inhibitor of cellular migration (Carmeliet 1995, Lijnen 1996).

In the following section, the single substances taking part in the fibrinolytic system, t-PA, u-PA, PAI-1, PAI-2 and non-specific activators and inhibitors, will be presented. Interactions with fibrin, complex formation and clearance will be discussed at the end of this section.

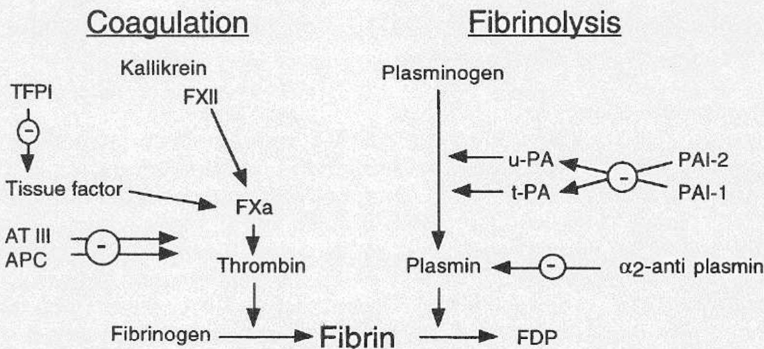


Fig. 2 Schematic figure of coagulation and fibrinolysis.

Tissue plasminogen activator (t-PA)

A glycoprotein with Mr of 65 000, belongs to the serine protease family. Kringle and finger domains in the molecule give t-PA specific fibrin affinity. t-PA is secreted primarily by endothelial cells although synthesis has also been demonstrated by macrophages, mesothelium and various tumour cells (Risberg 1986, Hinsbergh 1987, Hart 1988, Falkenberg 1996). Consequently, the capillary bed, representing a

huge endothelial surface of about 1000 m^2 is the main source of t-PA (Chandler 1991, 1996, Schrauwen 1994).

t-PA is released from the cell as a single-chain molecule (sct-PA). Plasmin could cleave and convert single-chain to two-chain t-PA (tct-PA). As they have different kinetics, both sct-PA and tct-PA are active plasminogen activators (Rånby 1982, Kluft 1994, Rijken 1995). t-PA could be bound both to fibrin (as will be discussed later) and to cell surfaces. At the cell membrane, t-PA effectively activates surface-bound plasminogen to plasmin. Furthermore, surface t-PA and plasmin have been demonstrated to be protected from inactivation by PAI and α_2-antiplasmin respectively. Consequently, high local fibrinolytic activity could be maintained at the surface (Haijjar-86, Bu 1994, Plow 1995, Redlitz 1995).

Release / Regulation, t-PA

Two types of stimulated cellular t-PA release have been described; a) a rapid – short and b) a slow – continuous release (Tranquille 1990, Kooistra 1994). In vitro, the rapid t-PA release has been demonstrated to be proportional to the slower new-synthesis release (v Eijnden-Schrauwen 1995). In contrast to PAI-1, t-PA demonstrates only a weak circadian variation and a limited acute-phase protein response (Chandler1991).

a) The rapid, albeit limited, release originates from pre-synthesized, cell-stored t-PA (Tranquille 1989). Endothelial cells could bind t-PA on their surfaces (Redlitz 1995, Cheng 1996), but even intracellular storage vesicles have been predicted (Kooistra 1994). Probably, separate pools with different regulation exist, as the post-release desensitization has revealed itself to be substance-specific (Kooistra 1990). In vitro, the cellular pools have been demonstrated to be quite small, but there are indications that they could contain much more t-PA in vivo (Tranquille 1989, Padró 1990, Giles 1990, v Eijnden-Schrauwen 1995). The rapid release could be induced by agonists connected to the protein kinase C /Ca^{++} system such as thrombin, histamine, substance P, endothelin, bradykinin, epinephrine and PAF (Zhu 1989, Tranquille 1990, Chandler 1992, Kooistra 1994, Jern 1994). Cyclic nucleotides (cAMP, cGMP) could modulate but not induce acute release (Tranquille 1993). t-PA increased by exercise, is induced by a combination of epinephrine-increased release and a decreased hepatic clearance (Chandler 1993).

b) The slower but quantitatively larger t-PA release from the endothelium has been found to be preceded by a lag-phase of protein synthesis. Consequently, this release could be controlled at the transcription and translation levels. Several substances have been demonstrated to increase this synthesis-dependent release: thrombin and histamine (Hanss 1987, Dichek 1989, Francis 1989, Grülich-Henn 1990), retinoids (Kooistra 1994), sheer stress (Diamond 1989, Iba 1991) and lipids such as butyrate (Kooistra 1987). On the other hand, no effect has been observed following exposure to endotoxin or cytokines (Bevilacqua 1986, Hanss 1987, Schleef 1988). In human endothelium, neither steroids nor bFGF have proved to have any effect on t-PA production (Kooistra 1994).

Urokinase (u-PA)

Urokinase is a glycosylated serine protease with a Mr of about 54 000. Compared with t-PA, the molecule, contains only one kringle and no finger domain and, consequently, has no fibrin specificity. u-PA is secreted from the cell as a inactive single-chain molecule scu-PA. Plasmin, kallikrein, or FXIIa could cleave scu-PA and create active two-chain tcu-PA. Only the active tcu-PA could rapidly form an inactivating complex with PAI-1 or PAI-2. tcu-PA is also inactivated, but at a much

lower rate, by α_2-antiplasmin, α_2-macroglobulin and C1-inhibitor. In plasma, the half-life of tcu-PA is about 8 minutes and almost all the u-PA is in the non-inhibited, non-active scu-PA form. u-PA production has been reported from several different cell types: endothelium, fibroblast, smooth muscle cells, epithelial cells, pneumocyter, trophobasts, monocytes and various tumour cells (Hart 1988, Duffy 1993, Vassalli 1994, Danø 1994, Rijken 1995). In the non-perturburated state, in vitro u-PA has been found only in the kidney and lung tissue (Quax 1990).

uPA production could be increased by several factors, interacting both transcriptionally and post-transcriptionally (Bessner 1996). Growth factors, phorbolester, cytokines and coagulation factor such as thrombin have all been demonstrated to increase u-PA (Pepper 1993, Blasi 1993, v Hinsbergh 1994, Gualandris 1995).

Several cells have specialised u-PA receptors (u-PAR) on their surfaces. The receptor binds the EGF domain on single- or two-chain u-PA. Inactive scu-PA could be converted to tcu-PA at a much higher rate when bound to the receptor. u-PAR-bound u-PA could rapidly cleave cell-bound plasminogen into active cell-bound plasmin. The cell could regulate the position and affinity of u-PAR on its surface and also determine when this should happen. This ability could create a migrating, invasive potential. Many tumour cells express u-PAR on their own surfaces but stimulate other stroma cells to produce u-PA (Blasi 1993, 1994, Danø 1994). In general, the u-PAR is upregulated by cytokines, TGFβ, phorbolester, bFGF (Pepper 1993, Blasi 1994, Vassalli 1994, Lund 1995, Bessner 1996). In contrast to t-PA, cell-bound tcu-PA could be inactivated by PAI-1 or PAI-2. The inactive tcu-PA/PAI /u-PAR complex is internalized into the cell by the low-density lipoprotein (LDL) receptor-related protein (LRP). The complex is fragmented and the receptor could re-cycle to the surface (Blasi 1993, Strickland 1994).

Other fibrinolytic activators

The contact phase of coagulation represented by factor XII and kallikrein could contribute to fibrinolysis. This pathway is named "the intrinsic activation". The activity is generated through several routes: kallikrein could convert scu-PA to tcu-PA that will release bradykinin or kallikrein could also activate an non-identified plasminogen activator. The physiological importance of this system is probably very limited. The effect has been demonstrated only at massive activation of the contact system using extra corporal circulation or at C1-esterase inhibitor deficiency (Binnema 1990, Kluft 1994, Rijken 1995, Chandler 1996).

Transgenic animals with a double knock-out of both t-PA and u-PA or single knock-out affecting plasminogen could develop and survive. These data call upon the existence of other non-plasmin-mediated fibrinolytic principles (Carmeliet 1995). Matrix metalloproteinaser (MMP), normally interacting with plasmin in basal lamina degradation, could be an alternative fibrinolytic pathway (Lijnen 1996, v Leeuwen 1996). By secretion of elastase and cathepsin G, polymorphnucleated neutrophiles and macrophages could also create a non-plasmin fibrinolysis (Magnatti 1993, Soo 1996).

Plasminogen activator inhibitor type-1 (PAI-1)

Plasminogen activator inhibitor type-1 (PAI-1) is a glycoprotein in the serpin inhibitor family with Mr 52 000. PAI-1 is a fast inhibitor of sct-PA, tct-PA and tcu-PA but not of scu-PA (Sprengers 1987, Hart 1988, Loskutoff 1989, Krishnamurti 1992).

PAI-1 has been demonstrated to be secreted from several types of cell including endothelial cells, hepatocytes, smooth muscle cells, fibroblasts, endometrial cells, synovial cells, adiposal cells and megakaryocytes (Loskutoff 1989, Krishnamurti

1992, Thornton 1995). There has been diverging data demonstrating the source of PAI-1 in vivo. Freshly-harvested endothelial cells produce low levels of PAI-1, which rise considerably when cultured (vd Berg 1988). The production of PAI-1 in vitro seems to be much higher than the calculated production in vivo (Chandler 1996). A substantial PAI-1 secretion has been measured over the hepatic circulation (Brommer 1988), compared with only a small release over the peripheral capillary bed (Kreb 1990, Jern 1994). On the other hand, immunological and in situ hybridisation methods have shown PAI-1 to be co-localised with endothelial cells throughout the entire body (Loskutoff 1991, Simpson 1991, Keeton 1993, Falkenberg 1996). In vitro PAI-1 production from endothelium was found to decrease when co-cultured with smooth muscle cells (Christ 1992). To sum up, the in vitro situation probably represents a state of non-specific upregulation.

Active – Latent PAI-1, Vitronectin

PAI-1 is secreted from the cell as an active glycosylated molecule. In a free-fluid phase, the active molecule, with a half-life of 2-3 hours, spontaneously undergoes tertiary conformation changes and becomes inactive "latent PAI-1". "Latent PAI-1" could be reactivated under laboratory conditions. However, in vivo, "latent PAI-1" is thought to be in an irreversible inactive form (Levin 1986, Kooistra 1986, Lambers 1987, Hekman 1988, Boström 1990). Platelets contain a great pool of PAI-1 that will be liberated as the platelets de-granulate. A very high percentage of this megakaryocyte-synthesized and platelets-stored PAI-1 is in the latent inactive form. Nevertheless, platelet-rich clots are semi-resistant to t-PA induced fibrinolysis, probably because of PAI-1 activity from de-granulating platelets (Kruithof 1987, Declerch 1988, Simpson 1990, Handt 1996, Robbie 1996).

Active PAI-1 binds to the liver synthesized macromolecule vitronectin. This binding stabilises the active form of the PAI-1 molecule. Vitronectin, binding PAI-1, exists both in plasma and as an integrated part of the extracellular matrix (Wiman 1988, Mimuro 1989a, 1989b). In vitro, the small matrix-bound active PAI-1 pool (1-2 ng/cm^2) has been found to represent an exchangeable and potentially biologically active part of the matrix (Levin 1987, Mimuro 1987, 1989a, Schleef 1990, v Leeuwen 1996). On the other hand, no acute PAI-1 release as for t-PA has been detected (Schleef 1988, Diamond 1989, Keber 1990, Jern 1994). Vitronectin could also form trimer-complexes with PAI-1 and thrombin or with heparin and thrombin. Complex formation significantly inhibits thrombin-induced APC activation (Rijken 1995).

Release / Regulation, PAI-1

After stimulation, a lag-phase of several hours with DNA transcription, translation and glucosylation precedes increased PAI-1 secretion from the cell (Laiho 1987, Schleef 1988, Medina 1989, Loskutoff 1991). Several substances have been reported as influencing the PAI-1production in vitro:

Phospholipase C, activated by substances such as thrombin and histamine (Gelehrter 1986, Hanss 1987, Dichek 1989, Francis 1989, Grulich-Henn 1990).

Inflammatory mediators such as interleukin-1 (IL-1) (Median 1989, Beviliacqua 1986, Schleef 1988) and tumor necrosis factor (TNF_α) (vd Berg 1988, Median 1989, Sawdey 1989, v Hinsbergh 1990, 1994), an effect that could be potentiated by interferon-γ (Arnman 1995). Several studies have demonstrated that endotoxin (LPS) induces PAI-1 production. However, there have been great differences between reports from in vivo and in vitro experiments and also between sera and non-sera in vitro experiments (Colucci 1985, Hanss 1987, vd Berg 1988, Median 1989, Sawdey 1989, 1991, Slivka 1991, Quax 1990).

Growth factors such as transforming growth factor-beta (TGFβ) have been reported by several authors to increase PAI-1 production (Laiho 1987, Keski-Oja 1988, Sawdey 1989, Slivka 1991) and endothelial cell growth factor (ECGF) to decrease PAI-1 production (Konkie 1988). In vitro, hepatocytes, but not endothelial cells, increase PAI-1 production when exposed to insulin or corticosteroides (Rehmentulla 1987, Heaton 1989, Loskutoff 1991). Matrix molecules underneath the cell could also influence PAI-1 production (Gillis 1995).

Sheer stress and physical exercise increased t-PA but had no effect on PAI-1 production (Diamond 1989, Chandler 1993).

In epidemiological studies, increased levels of PAI-1 have been correlated to ageing, smoking, general arteriosclerosis including myocardial infarction, diabetes, malignancy, hyperlipidemi, server liver disease and pregnancy. It has also been suggested that polymorphism in the gene influences plasma PAI-1 levels (Hamsten 1993, Takada 1994).

In general, PAI-1 shows a circadian variation with the highest concentrations in the early morning. PAI-1 also acts as an acute-phase protein increasing after trauma, operations and sepsis.

Plasminogen activator inhibitor type-2 (PAI-2)

PAI-2, a serine proteinase inhibitor in the serpin family. The molecule exists in two forms; intracellular non-glycosylated with a Mr of about 46 000 and an extracellular glycosylated form with a Mr of about 70 000. PAI-2 is mainly an inhibitor of tcu-PA. Non-fibrin-bound tct-PA could also be inhibited but this effect is probably not physiological (Åstedt 1985, 1987, Lecander 1986, Dear 1995). PAI-2 was initially isolated from placenta and plasma from pregnant women. It was demonstrated later to be produced from a wide range of cells. Monocytes, leukocytes, keratinocytes, granulosa cells, microglia, endothelium, and mesothelium cells all produce PAI-2. Several tumour cells secrete PAI-2 and, with some exceptions, a relationship between high PAI-2 levels and restricted metastatic potential has been found (Åstedt 1985, 1987, Lecander 1986, Dear 1995).

PAI-2 production is increased by cytokines (such as TNFα, IL1, IL-2), angiotensin II, phorbolester, and phosphatase inhibitors such as okadaic acid. Production is decreased by dexamethasone, retinoids, TGFβ, IL-4 and modulated by cyclic AMP. This pattern of regulation indicates both G-protein, protein kinase C, and tyrosine kinase signal transduction.

Extracellular PAI-2 could modify cell surface fibrinolysis and cellular migration. Being more resistant to oxidants than PAI-1, it may modulate inflammatory processes. The biological function of intracellular PAI-2 is still not quite clear. A role in differentiation, apoptosis and inflammatory response has been suggested. (Åstedt 1985, 1987, Lecander 1986, Rijken 1995, Dear 1995).

Other plasminogen activator inhibitors

In physiological conditions, PAI-1 and PAI-2 are the principal inhibitors to plasminogen activators owing to the high constant rate in complex formation. There are several other substances with a much lower PA inhibitory potential: α_2-macroglobulin, C1-inhibitor, α_2-antiplasmin, PAI-3 (APC-inhibitor), antitrombin III and protease nexin 1. These substances could serve as a second line of inhibition if, for example the capacity of PAI-1 and PAI-2 becomes overloaded during thrombolysis therapy (Sprengers 1987, Hart 1988, Rijken 1995).

PA and PAI interactions

Fibrin interaction

Both plasminogen and t-PA could be bound to fibrin. This fibrin co-binding increases t-PA conversion of plasminogen to plasmin approximately 200-400 times. Plasmin is also partially protected from α_2-antiplasmin inhibition when localised to the fibrin surface. Local plasmin proteolysis, in a positive feedback loop, further degrades Glu-plasminogen to Lys-plasminogen and single chain t-PA, u-PA to two-chain t-PA, u-PA respectively. The fibrin specificity is performed by lysine bindings on the kringel and finger domains that exist on plasminogen, t-PA, and PAI-1, but not on the u-PA molecule. Consequently, the PAI-1 (even latent) and t-PA/PAI-1 complex could compete with plasminogen and t-PA for these bindings. Further lysine-rich substances such as lipoprotein(a) or therapeutically-added tranexamic acid could block the bindings and thereby decrease fibrin adhesion and degradation (Rånby 1982, Mosesson 1990, Redlitz 1995, Blombäck 1996). The interaction between fibrin and u-PA is complicated. Partially plasmin-degraded fibrin exposed new binding sites for plasminogen. Bound plasminogen changes conformation and becomes attractive for scu-PA binding. scu-PA, still being inactive, could be cleaved and activated by plasmin (Fleury 1993, Chandler 1996).

Endothelial cells that have been covered at their luminal surface with fibrin increase t-PA and decrease PAI-1 production (Fukao 1995). The cells also change phenotype into a synthesizing, migrating form (Kadish 1979, Soo 1996). Endothelial produced PAI-1 could has been demonstrated to infiltrate and delay fibrinolysis of fibrin clots in (Handt 1994, 1996).

Complex formation / Clearance

In the circulation, the fibrinolytic activity is mainly determined by the level of active t-PA. The free t-PA activity could be rapidly inhibited through complex formation, normally with PAI-1. In a two-phase reaction, t-PA (or tcu-PA) first binds to and then cleaves and becomes catched into an inactive 1:1 complex with the PAI molecule. The enzyme reaction between t-PA and PAI-1 has a very high rate constant, but as the concentrations of the substances in plasma are low, a steady state with a considerable amount of free active t-PA is created (Sprengers 1987, Hekman 1988). At a physiologically high PAI-1 concentration, (300 pmol/l) only 10% of plasma t-PA is active, rising to 20-50% free t-PA when the PAI-1 concentration is in the lower range (<100 pmol/l) (Chandler 1991).

The hepatic clearance of t-PA, PAI-1 and the t-PA/PAI-1 complex is rather rapid owing to several different receptor mechanisms (Redlitz 1995). The elimination is a direct function of the hepatic blood flow. For the t-PA/PAI-1 complex, half-life is approximately 3-5 minutes. Free t-PA has an even faster hepatic elimination and also rapid PAI-1 inactivation in the circulation, giving a normal half-life of only about 1 minute. The "hepatic" half-life of active PAI-1 is about 10 minutes and together with complex binding at normal t-PA levels gives a total half-life of about 5 minutes (Sprengers 1987, Brommer 1988, Chandler 1996). Disturbed hepatic elimination could influence the fibrinolytic activity in plasma. Hepatic dysfunction, such as cirrhosis or prolonged bile stasis, results in an increased fibrinolysis because of a combination of decreased t-PA clearance and reduced production of PAI-1 and α_2-antiplasmin (Huber 1991).

This section has summarized the fibrinolytic system. The regulation, turnover and proposed biological function of the substances involved in the cascade has been discussed. More about the intracellular regulation of fibrinolysis will be described in the corresponding parts in the result and discussion sections.

Intracellular signal transduction

An extracellular signal coming to the cell has to pass through the cellular membrane and continue through the integrating net of transducting pathways before the message finally reaches a specific intracellular effector system.

A transmembrane receptor-protein transducts the signal through the cell membrane. Each receptor type is unique for a specific extracellular substance. When a receptor binds an agonist, the conformation of the receptor molecule is changed, uncovering new catalysing sites of the intracellular part. Depending on the type of catalytic activity and intracellular micro-environment, the receptor-signal could be linked to different transduction pathways. The transducting pathways are cascades of subsequent enzyme reactions bringing the signal to the effector system, being an enzyme system or a gene promotor region. Several points of interaction between the different pathways exists ("cross-talk"), modulating the original signal (Hausdorff 1990, Brass 1993, Seger 1995, Post 1996).

In the following section, parts of the signal transduction systems will be discussed. The presentation has been indexed in subdivisions:

- A) Main transduction pathways:
  - 1)The GTP-binding protein systems
    - a) The adenylate cyclase system
    - b) The phospholiphase C/D - protein kinase C system
  - 2) The mitogen-activated protein (MAP) kinase system
- B) General intracellular signal systems:
  - 1) Ca^{++} system
  - 2) Transcription factors
- C) Intracellular effector systems:
  - 1) The fast enzyme system, Arachidonic acid - Prostacyclin
  - 2) The slow gene-transcription system, t-PA and PAI-1

Main transduction pathways

GTP-binding protein systems

Receptors linked to the adenylate cyclase or phospholiphase C/D system, belong to the seven transmembrane-spanning receptor family. These receptors transduct signals further through the membrane bond GTP-binding protein system. In the non-activated state, the intracellular part of the receptor molecule is bound to a protein dimer, the $\beta\gamma$-subunit. Another protein, the α-subunit, is linked to the $\beta\gamma$-dimer, creating a trimer. The α-subunit could also bind GDP in this state. When the receptor is activated, it catalyses the α-subunit to release GDP and instead binds GTP. The GTP-bound α-subunit has low affinity for the $\beta\gamma$-dimer and dissociates. The $\beta\gamma$-subunit is also now released from the receptor. The free α-unit or even high concentrations of the free $\beta\gamma$-dimer could interact with different intracellular enzymes. The active α-subunit is auto-inactivated, as GTP is hydrolysed to GDP; a reaction that could be increased in a negative feed back loop by several effector enzymes. After being hydrolysed to GDP the $\alpha\beta\gamma$-trimer could be reformed and be ready for new signals to be transducted.

There are 22 different α-subunits, 7 different γ-subunits and 4 different β-subunits identified. Different types of subunit could induce different intracellular responses, creating an enormous potential of combinations. A single activated receptor could

dissociate many α / $\beta\gamma$ -subunits, sometimes even of different types. Hence the GTP-binding protein system permits transduction, potentiation and diverging of the receptor signal (Hausdorff 1990, Birnbauer 1992, Hepler 1992, Bernstein 1992).

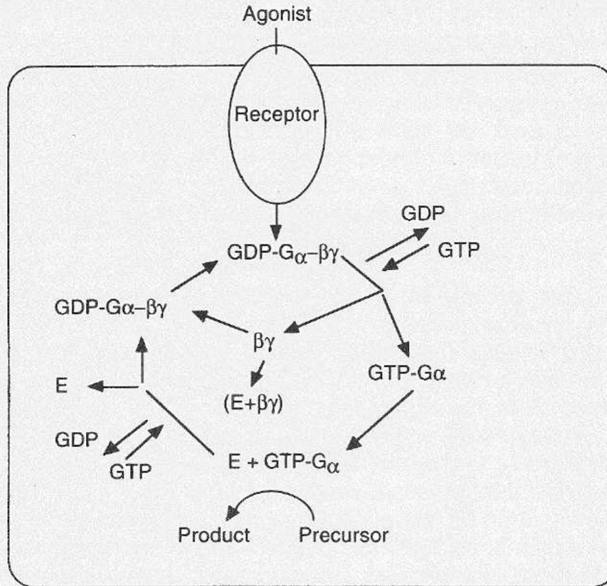


Fig 3. Receptors linked to the adenylate cyclase or phospholiphase C/D system, transduct signals through the membrane bond GTP-binding protein system to the effector system (E).

The adenylate cyclase system

Many cell-surface receptors, for example the β-adrenoceptor activates GTP-binding protein α-subunits of the s- type, $G_{\alpha s}$-protein. The α-adrenoceptor activates the $G_{\alpha i}$-protein by dissociating it from the $\beta\gamma$-dimer. $G_{\alpha s}$ activates and $G_{\alpha i}$ inactivates the membrane-bound enzyme adenylate cyclase (AC) (Sibley 1985, Hausdorff 1990, McEwan 1990). The effect of the free $\beta\gamma$-dimer could be different, depending on the cell-specific type of AC-isomer (Iyengar 1993, Manolopoulos 1995b). Other non-AC-transducted functions have also been reported for the $G_{\alpha s}$ /$G_{\alpha i}$ /$\beta\gamma$ units, although not completely characterized in endothelium (Barber 1989, Schubert 1989, Yatani 1989, Limbird 1990, Williams 1995).

AC converts intracellular AMP to cyclic AMP (cAMP). cAMP activates protein kinase A (PKA). This is a kinase with several intracellular effector substrates. The C-terminal fragment of PKA could also act as a transcription factor on CREB (Karin 1992, Hunter 1995) (se below). cAMP is inactivated by enzymes with phosphodiesterase (PDE) capacity (Hall 1989). The desensitization of the β-adrenoceptors will be discussed in the prostacyclin section under Discussion.

The phospholiphase C/D - protein kinase C system

In addition, in this system the surface receptors are of the seven transmembrane-spanning family, transmitting signals through the GTP-protein binding system. The effector enzymes, phospholipase C (PLC) and phospholipase D (PLD), are

associated with the cellular inner-membrane (Exton 1985, Liscovitch 1992, Roberts 1996).

PLC cleaves the membrane lipid phosphatidyl-inositol-biphosphate (PIP_2), releasing inositol-trisphosphate (IP_3) and diacylglycerol (DAG) into the cytosol (Lee 1995). IP_3 binds specifically to a receptor on the endoplasmatic reticulum, releasing Ca^{++} to the cytosol. DAG could activate the multi-potent enzyme protein kinase C (PKC) or be further converted to arachidonic acid (AA) (Nishizuka 1995).

PLD, being more unexplored, cleaves phosphatidyl-inositol or phosphatidyl-choline to the second messenger phosphatic acid (PA). By an enzymatic reaction PA could be further converted to DAG, and this pathway probably represents the long-lasting DAG phase after receptor stimulation (Exton 1990, Garcia 1992, Sozzani 1992).

The mitogen-activated protein (MAP) kinase system

The amino acids, tyrosine, serine and theonine, could exist in two forms; phosphorylated, catalysed by kinases, or dephosphorylated, catalysed by phosphatases. Many proteins with these amino acids in their sequence could change conformation when phosphorylated. This three-dimensional shift could be accompanied by the unmasking of active enzyme sites. This newly-activated enzyme, if a kinase, could activate another pro-kinase and create a cascade reaction. To balance the system, phosphatases inactivate the kinases by dephosphorylering.

The MAP kinase cascade or "extracellular signal-regulated kinases" (ERK) is so called for historical reasons, as the MAP kinase was the first enzyme in the cascade to be discovered. Surface receptors for several growth factors, cytokines and adhesion molecules, have intracellular regions that directly or indirectly could express kinase activity (Cobb 1991, Seger 1995, Hunter 1995). In a complex net of interactions, the activated receptor could interact with cell membrane, cytoskeleton or focal adhesion proteins through intermediating proteins (Grb_2, SOS, Shr, src-family). The signal is transducted to the "small GTP-binding protein", Ras, a common converging point in the cascade. In a subsequent series of reactions, Ras activates the kinase Raf-1, activating the kinase MEK, activating MAP kinase. MAP kinase is a multipotent enzyme phosphorylating transcription factors, cell-cycle regulators, cyto-skeletal proteins and phospholipid A_2. Several points of interaction exist between the MAP kinase cascade and GTP-protein-coupled receptors (Lin 1993, v Corven 1993, Johnson 1994, v Biesen 1995, Erpel 1995, Post 1996, Bokoch 1996). Raf-1 is a kinase regulated by cAMP/PKA (Johnson 1994, Post 1996), and PKC (Kolch 1993, Nishizuka 1995).

General intracellular signal systems:

Ca^{++} system

Ca^{++}, an important co-factor, acts both extra and intracellularly. Inside the cell, Ca^{++} is a fast and potent second messenger for many enzyme reactions, PLA_2, nitric oxide synthases (NOS) and myosin light chain kinase. The free cytosolic Ca^{++} concentration is normally very low. Ca^{++} is stored by active ion-pumps into the endo-plasmatic reticulum (ER) or excreted extracellularly. Phospholipase C -stimulated IP_3 could activate a receptor on the ER (Exton 1985, Adams 1989, Jacob 1990, Clapham 1995). The activated ER-receptor releases storaged Ca^{++} into the cytosol. The liberated Ca^{++} has been demonstrated to increase further Ca^{++} release, creating a fast wave of increased cytosolic Ca^{++} concentration which sweeps over the cell.

This rapid Ca^{++} flux is referred to the "initial high peak". The peak is followed by a lower but sustained "plateau phase", important both for enzyme activation and also for the refilling of intracellular Ca^{++} storage. This phase represents extracellular Ca^{++} influx through several differently regulated transmembrane channels. The influx depends both on the receptor-controlled probability of channel opening and the driving ion force created by the membrane potential (Adams 1989, Jacob 1990, Bezprozvanny 1991, Miyazaki 1995). Hence factors controlling transmembrane ion flux (K^+, Na^+, Mg^{++}, H^+) that could change the membrane potential (Sigel 1991, Graier 1993) or pH (Sweatt 1986, Madshus 1988, Kitazono 1989) have implications for the cytosolic Ca^{++} concentration. Phosphorylating kinases could interfere with intracellular Ca^{++} by regulating transmembrane influx and refilling receptor-emptied ER storage (Fleming 1995). To sum up, Ca^{++} is an important intracellular activator. The cytosolic Ca^{++} concentration is the integrated sum of several cellular control mechanisms.

Transcription Factors

Transcription factors (TF´s) are specialised molecules which transduct signals to the gene-promotor regions (Karin 1992, Hunter 1992, Hill 1995, Jans 1995). Some TF´s are primarily located in the cytosol and transported to the nucleus when activated, whereas others are strictly intranuclear. TF´s have different sequences for transporting the molecule into the nucleus for binding a specific part of the DNA and for activating DNA. Phosphorylation or de-phosphorylation of these separated sequences has been demonstrated to be used by different TF´s to up- or down-regulate gene activity. These regulatory mechanisms could explain why, for example, MAP kinase could induce a diverging effect with different TF´s. Another principal influencing gene-promoter has been described for some hormones and growth factors where the entire agonist could be internalized and transported to the nucleus, where it could bind the gene (Jans 1995).

The CRE (cAMP Response Element) sequence on DNA could be activated by phosphorylated CREB (CRE Binding protein) in complex with a member of the ATF family (ATF= Activating Transcription Factor). When cAMP activates cytosolic PKA, the C-terminal part of PKA will be released and transported into the nucleus. By phosphorylating CREB, the catalytic C-terminal initiates conformation changes and activation of the CRE sequence. Simultaneously, the C-terminal activates CREB-inactivating phosphatases, shutting off the signal (Karin 1992, Wang 1992, Hunter 1992, 1995, Hill 1995).

The TPA responsive element (TRE) sequence of the DNA could bind transduction factors in the AP-1 (Activator Protein-1) family, which are different dimers of jun and fos. These TF´s could be activated or deactivated in a complex net of interactions by PKC or kinases in the MAP cascade. The total activation is a function both of increased transcription of jun/fos (early response genes) and a modulated activation of latent jun. (Karin 1992, Wang 1992).

Other kinases such as $MAP/ERK^{42/45}$ and Rsk^{90} could also be translocated into the nucleus, phosphorylating intranuclear transcription factors (Johnson 1994).

Nuclear Factor kappa Beta (NFκB) represents a type of TF that when bound to an inhibiting protein (IκB) is primarily located to the cytosol. Different "inflammatory" signals such as cytokines (IL-1, TNF_{α}), endotoxin or PKC, could degrade the inhibitor (IκB), releasing NFκB. The "free" NFκB will then migrate into the nucleus, where it could influence several different genes and induce the cellular "inflam-

matory" response. The degradation of IκB could be inhibited by serine protease inhibitors (Hill 1995, Thanos 1995). Hypothetically, as PAI-2 induction is known to inhibit the inflammatory effect of TNFα (Dear 1995), NFκB / IκB might be one of the targets for intracellular PAI-2.

Intracellular effector systems

Finally, the modulated intracellular signal reaches effector systems. The product of these systems could be cell-surface-bound or secreted substances or a cytoskeleton rearrangement with or without a permeability shift. The effector system could react fast when the signal influence the activity of an already synthetized product, e.g. an enzyme. On the other hand, when the final product depends on a new protein synthesis, the effector system has to have a lag-phase of transcription and translation. As this thesis focuses on the rapidly produced lipid metabolite prostacyclin and on the lag-phase synthetizsed glycoproteins t-PA and PAI-1, the synthesis pathways of this substances will be discussed / presented.

The fast enzyme system, Arachidonic acid - Prostacyclin

Arachidonic acid (AA) is created from membrane bound lipids, especially phosphatedyl-choline, cleaved by the enzyme, phospholipid A_2 (PLA_2). To a minor degree, intracellular DAG could also act as a source for AA. Free AA is rapidly converted to some of several different eicosanoids, (prostaglandins, leukotrienes, lipoxins). The synthesis pathway depends on the actual cellular access of enzymes. In endothelial cells, AA is converted by cyclooxygenase (COX) to the prostaglandin precursor, PGH_2 (Dusting 1990, Liscovitch 1992, Gerritsen 1996, Roberts 1996).

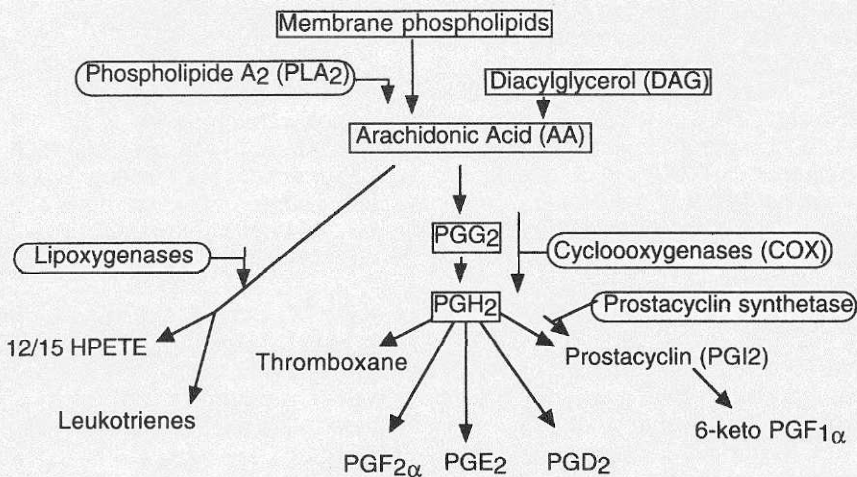


Fig 4. Metabolic pathways of eicosanoid biosynthesis.

The enzyme COX is made up of two parts; a peroxidase and a cyclooxygenase. A intermediator in the synthesis (PGG_2) irreversibly inactivates COX and a new protein synthesis is needed to recover COX activity. There are different types of COX. COX-1 is responsible for constitutive prostacyclin production and COX-2 for cytokin-induced production (Shimizu 1994, Gerritsen 1996).

In endothelium, PGH_2 is preferably converted to prostacyclin (PGI_2) by PGI_2-synthetase, although a small amount of prostaglandin E_2 (PGE_2) and a trace amount of thromboxane A_2 are also produced.

The rate-limiting step (in the production of PGI_2) is the activity of the enzyme PLA_2. An important regulation of PLA_2 is mediated by Ca^{++}, bringing cytosolic PLA_2 in contact with the membrane substrates. The activity of PLA_2 could also be regulated by receptor-induced GTP-binding proteins, MAP-kinases (Lin 1993, Johnson 1994, Seger 1995) and PKC (Nishizuka 1995).

The slow gene-transcription system, t-PA and PAI-1

t-PA and PAI-1 molecules could be secreted from the cell as a result of a series of cellular activities: activation of the promotor, transcription of DNA, translation to form the protein core, glycosylation in Golgi and, finally, excretion of the glycoprotein. As discussed earlier, several extracellular stimuli could induce t-PA and/or PAI-1 production from endothelial cells. Briefly, inflammatory mediators, growth factors, adhesion substances and sheer stress use the MAP- kinase systems, whereas PLC activating substances use the PKC system. However, the signal transduction net-work is complicated and has not been fully explored (Loskutoff 1989, Kooistra 1994, Chandler 1996). Regulation of the synthesis occurs mainly pre-transcriptionally. Post-transcriptional modulation regulating mRNA stability, Golgi sorting and phosphorylation is probably not that important in t-PA and PAI-1 production, as it is, for example, in u-PA synthesis (Loskutoff 1991, Gualandris 1995).

The promotor area is the starting and control point of a gene. Several enhancer and repressor elements with affinity to specific transduction factors could be located in this DNA sequence. Every gene has a unique set of responding elements to control the transcription of mRNA for the coded protein (Medcalf 1990, Loskutoff 1991, Ohlsson 1992). DNA regions and the function of several transcription factors, hormones and kinases have been described, by constructing and examining short sequences of the promotor. The results of these "split" experiments have sometimes been difficult to extrapolate to the in vivo situation, probably because of the occurrence of "transcription complex". Different TF´s could "aggregate" into large "transcription complexes" creating a specific three-dimensional conformation that could fit into and fold the DNA chain. This arrangement will permit the complex to simultaneously influence several separate regions on the promotor (Collins 1995, Edmondson 1996).

AIMS OF THE STUDY

The general aim of the present investigation was to clarify the regulation of anti-thrombogenetic factors in endothelial cells and particularly in relation to:

- the influence of adrenergic control in endothelial prostacyclin production,
- the regulation of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1) production in endothelial cells,
- the differences in regulation of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1) production in endothelial cells of different origin.

To achieve this, the following experiments were performed:

a. explorative studies, using agonists and antagonists representing different signal transduction pathways,
b. cross-talking studies, examining the effect of adenylate cyclase and protein kinase C inhibition on endotoxin, phorbolester and thrombin-induced production,
c. thrombin signal transduction studies, exploring GTP-binding protein and tyrosine kinase,
d. comparative experiments on cultured human endothelial cells from umbilical veins and adult saphenous veins.

MATERIALS and METHODS

Drugs

The following substances and concentrations have been used in the experiments. Unless specified otherwise, all the substances were from Sigma Chemicals, Sweden.

Forskolin, 1uM - 500uM,
a direct adenylate cyclase activator, increasing the intracellular level of cAMP (Keen 1992, Iyengar 1993). Stock solutions had to be dissolved in DMSO, the maximal final concentration 0,04% being atoxic.

Isoprenalin (= isoproterenol), 10^{-9} - 10^{-5} M,
a β_1- and β_2-adrenoceptor agonist.

Ephedrine, 10^{-8} - 10^{-4} M,
a β- and α-adrenoceptor agonist.

Isobutylmetylxantin (IBMX), 10^{-3} M,
a general phosphodiesterase (PDE) inhibitor, preventing cAMP degradation (Fredholm 1980, Hall 1989).

Propranolol, 10^{-5} M,
a β_1- and β_2-adrenoceptor antagonist. Propranolol has no intrinsic sympathetic activity (ISA) but has membrane-stabilising capacity.

Metoprolol, 10^{-5} M, (Astra Hässle, Sweden)
a semi-selective β_1-adrenoceptor antagonist with a dose-dependent β_2-receptor agonism. Metoprolol has no ISA and low membrane-stabilising capacity. Metoprolol isomers: S-metoprolol with high and R-metoprolol with low β_1-adrenoceptor antagonist effect, at the selected concentration (Harron 1981, Sandberg 1988, Wahlund 1990).

Na-nitroprusside (NP), 10^{-9} - 10^{-5} M,
a nitric oxide (NO) donator which stimulates the intracellular enzyme guanylate cyclase to produce cGMP. NP is unstable in bright light and the cell dishes had to be protected during the experiments (Harrison 1993).

Endothelin-1 (ET-1), 10^{-12} - 10^{-8} M (Peptide Institute INC, Japan),
a polypeptide with an endothelial ET_{B1} surface-receptor, linked to membrane G-proteins, PLC and Ca^{++} channels (Masaki 1991, Eguchi 1993).

Endotoxin (lipopolysaccharide LPS), 10^{-3} - 10ug/ml,
from E. Coli serotype 0111:B4. Unknown transduction inducing intracellular phosphorylation. In vivo, probably most of the effect secondary to cytokine induction (Morrisson 1993, Müller 1993, Redl 1993).

Phorbol 12-myristate 13-acetate (PMA), 1ng/ml-10ug/ml (=1,62nM - 16,2uM), producing a protracted activation of proteinkinase C (PKC) (Nishizuka 1995).

H7 (1-(5-isoquinolinylsulfonyl)-2-metyl-piperazine), 5uM - 100uM,
a dose-dependent, semi-selective PKC inhibitor at high concentration H7 can also inhibit cAMP (PKA) and cGMP-dependent protein kinase (Hidaka 1987). Stock solutions had to be dissolved in DMSO, with a maximal final concentration 0,01%, which is atoxic.

α-Thrombin, 10^{-3} - 10U/ml,
active thrombin from human plasma, without plasmin or plasminogen contents.

Pertussis toxin, 100ng/ml,
from Bordetella pertussis, by catalysing transfer of the ADP-ribose moiety of NAD to the α-subunit of the $G_{\alpha i}$-protein inhibits the $G_{\alpha i}$-protein in intact cells (Garcia 1991, Kaslow 1992).

Genistein, 2.5, 10, 40ug/ml,
a competitive ATP inhibitor, rather specific for tyrosine kinase, which blocks the transduction of signals in the MAP-kinase cascade, the IC_{50} being about 15 times lower for MAP-kinase than for protein kinase A or C (Akiyama 1991). IC_{50} for genistein decreased significantly with a low serum concentration (Hawker 1994), as used in the present study.

Stock solutions had to be dissolved in DMSO, with a maximal final concentration 0,065%, which is atoxic.

Sodium ortho-Vanadate, 30, 100uM,

an inhibitor of tyrosine-phosphatases (Gordon 1991). Stock solutions had to be warmed and alkalized; final test solutions had normal pH.

Bovine serum albumin (BSA), 0.1% in cellular medium,

to block unspecific binding sites in cell culture dishes. Endotoxin less than 0.1 ng/mg.

Dimethyl Sulfoxide (DMSO),

a detergent for hydrophobic substances. The final concentrations of DMSO never reached toxic levels (Levin 1989) and had no effect on t-PA or PAI-1 production (Data not presented).

See the following section for the content of the cell-culture medium.

Cell culture

Endothelial cells were harvested in accordance with Jaffe (Jaffe 1973a) by incubating human umbilical cords or adult human great saphenous veins with a 0.1% collagenase, Worthington type 1 solution (Sigma Chemicals, Sweden), at 37°C. The vessels were gently manipulated, and after 12 minutes the cell and enzyme solutions were flushed with serum containing growth medium (see below). The cell suspensions were washed twice and seeded on tissue culture dishes.

In paper I, primary cultures (passage 0) were used in the experiments and the cells were seeded directly on 1.9 cm^2 tissue culture dishes (Corning, NY, US) precoated with human fibronectin 10ug/cm^2 (Collaborative Research, US). In paper II - IV, primary cultures were seeded on 25 cm^2 or 75 cm^2 tissue culture dishes (Corning, NY, US) precoated with bovine gelatine 0.2% in cell culture medium (Sigma Chemicals, Sweden).

In all the experiments the cells were grown in M199 medium with Earl´s salt and 2.2 g/l sodium bicarbonate, supplemented with *L*-glutamine 0.234 mg/ml, penicillin 40 U/ml and streptomycin 40 ug/ml (all from Gibco, Sweden), heparin 100 ug/ml (Lövens, Sweden), ECGF 150 ug/ml prepared in accordance with Maciag (Maciag 1979) and 20% fetal calf serum (Kebo, Sweden). The cells were cultured at 37°C with 5% CO_2 supplementation. The endothelial origin of the cells in culture was confirmed both by their typical morphological cobblestone appearance and by positive factor VIII immunofluorescence reaction (Jaffe 1973b). For immuno-histochemistry, we used rabbit anti-human von Willebrand factor IgG and swine anti-rabbit FITC IgG (Dakopatts, Denmark).

In paper II - IV, the cells were subcultured by adding a solution of trypsin 0.05% and EDTA 0.02% (Gibco, Sweden). The cell solutions were washed twice with serum containing growth medium, and then seeded on 1.9 cm^2 tissue culture dishes (Corning, NY, US) precoated with bovine gelatine. In the experiments, quiescent endothelial cell cultures in passage 1 were used two days after reaching confluence

Biochemical Analysis

Prostacyclin

6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2 was analysed from the conditioned cell medium. A rabbit anti-6-keto-$PGF_{1\alpha}$ antibody was used in a I^{125}-RIA technique (Du Pont, NEN). The cross-reactivity has been reported to be below 0.8% (=PGE_1) for other eicosanoids. The range of the assay was 1 - 200pg/ml and dilutions of the samples were reported not to influence the measurement The coefficients of variation (CV) were described to be concentration-dependent, intra-assay (1.4%-5.2%) and inter-assay (6.8-12.4%).

t-PA and PAI-1

Commercially available enzyme-linked immunosorbent assays (ELISA) with polyclonal anti-human antibodies were used to determine the t-PA and PAI-1 antigen concentrations in the conditioned medium (TintElize t-PA and TintElize PAI-1, Biopool, Sweden).

The t-PA and PAI-1 antibody has been reported as detecting the different protein forms (single- or two-chain), active or latent, and the t-PA/PAI-1 complex with equal sensitivity. No cross-reactions with other cellular or cell medium substances have been detected. Down to an absorption of 0.050, the ranges of the assays were for t-PA 0.5 - 30ng/ml and for PAI-1 0.5 - 40ng/ml. Dilutions of the samples did not influence the measurement.

According to the manufacturer, the intra-assay coefficients of variation (CV) were 8% for t-PA and 10% for inter-assay. For PAI-1, corresponding values were 5% and 11% respectively.

Statistical Methods

Changes were expressed as percentages of the basal release within the same experiment.

In paper I, data were calculated with a two-way analysis of variance of logarithm values followed by multiple comparisons and are presented as ratios and 95% confidence intervals.

In paper II-IV, mean values are presented along with the 95% confidence intervals. Mean values exceeding the 95% confidence intervals in comparative groups were considered to be statistically significant. Differences were also tested for significance using the Wilcoxon rank sum test. $p<0.05$ was considered statistically significant. Significance testing, using either the 95% confidence intervals or Wilcoxon rank sum test, gave identical results.

Experimental design

Prostacyclin

In each experiment, primary cell cultures, originating from a single umbilical cord were used, seeded on multiple culture dishes. The cell dishes were handled gently throughout the experiment and placed in a CO_2 incubator during incubation.

The experiments were started by preincubating the cells for 15 minutes with blocking drugs (IBMX, metoprolol or propranolol) or pure medium M199, in accordance with the experimental design. After the preincubating period, the medium was replaced and the cell dishes exposed to 2 ml of new test solutions. The following combinations were added: a) pure medium M199, b) IBMX, c) IBMX + isoprenalin, d) IBMX + metoprolol, e) IBMX + propranolol, f) IBMX + isoprenalin + metoprolol, g) IBMX + isoprenalin + propranolol. The compositions were identical during the preincubation period, with the exception of isoprenalin, which was added only during the second incubation. All the test substances were dissolved in medium 199. This later incubation period lasted for 60 minutes. During the experimental period, the cell dishes were incubated at 37°C with 5% CO_2.

Samples of 0.2 ml were carefully collected from the conditioned medium. The samples were immediately mixed with an RIA-buffer containing 10ug/ml of indometacin before being frozen and stored at -70 °C until analysed.

t-PA / PAI-1

In each single experiment, cells derived from only one umbilical or saphenous vein were used. The cells, in passage 1, were plated on multiple cell-culture dishes and tested. Before the experiment, the culture dishes with endothelial cells were carefully washed twice with PBS-buffer.

In paper IV, inhibitors were added during a separate preincubation period of 4 hours for pertussis toxin and 1 hour for genistein or vanadate. The experiments were started by adding 2ml of the different test solutions to the culture dishes. The substances and concentrations were in accordance with the experimental design of the study. All the test substances were dissolved in a cell-growth medium without fetal calf serum or ECGF but supplemented with 0.1% bovine serum albumin (endotoxin less than 0.1 ng/mg). During the experimental period, the cell dishes were incubated at 37°C with 5% CO_2.

Samples were collected from the conditioned medium after 16 hours, and in paper II also after 24 hours´ incubation. The samples were immediately frozen, and stored at -70°C until analysed.

RESULTS

Prostacyclin (PGI_2) production

Basal production

Absolute basal release after 60 minutes of incubation ranged from 386 to 1554 pg/10^5 cells.

Stimulated prostacyclin production

In the following, data is expressed as percentages of basal release (indexed to 100%) in the same experiment, mean and 95% confidence interval of logaritmated values, n=numbers of cell dishes in the experiment.

After 60 minutes of incubation, the PGI_2 production was: IBMX (10^{-3} M) (109%, 96-123%, n=5) which was almost identically to "basal" cells. Isoprenalin (10^{-6} M) (155%, 104-233%, n=5) significantly increased PGI_2 production. Metoprolol (10^{-5} M) or propranolol (10^{-5} M) had no significant influence on PGI_2, producing 143%, 96-215%, n=5 and 160%, 53-266%, n=4 respectively. When isoprenalin (10^{-6} M) and metoprolol (10^{-5} M) were co-incubated, the PGI_2 production increased even further to 299%, 199-448%, n=5. A combination of isoprenalin (10^{-6} M) and propranolol (10^{-5} M) also increased PGI_2 production, 267%, 159-374%, n=4. (Fig 5.)

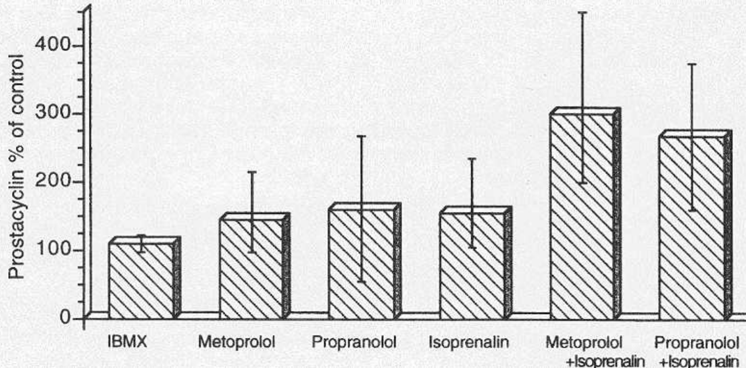


Fig 5. Samples from the conditioned medium of quiescent confluent cell cultures were collected after 60 minutes´ incubation with IBMX 10^{-3} M, metoprolol 10^{-5} M, propranolol 10^{-5} M, isoprenalin 10^{-6} M and analysed for the stable PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$. The results of the control dishes were set to 100%. Values are ratios related to control with 95% confidence intervals of logaritmated values, five separate experiments using different umbilical cords.

To further explore whether the β-adrenoceptor blocking capacity was of importance, metoprolol isomers with high (S-) or low (R-) blocking effect were tested.

Combinations of isoprenalin (10^{-6} M) and either the R- or S- metoprolol isomer (10^{-5} M) gave almost identical results. The PGI_2 production after 60 minutes` incubation was 193%, 121-306%, n=6 for R-metoprolol + isoprenalin and 198%, 125-315%, n=6 for S-metoprolol + isoprenalin. (Fig 6.)

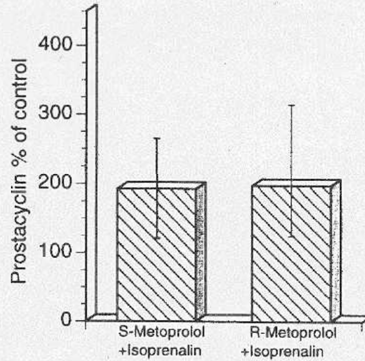


Fig 6. The effect of different metoprolol isomers 10^{-5} M, with high (S-) or low (R-) β-blocking effect, in combination with isoprenalin 10^{-6} M on the prostacyclin production. Samples from the conditioned medium of quiescent confluent cell cultures were collected after 60 minutes and analysed for the stable PGI_2 metabolite, $PGF_{1\alpha}$.The results of the control dishes were set to 100%. Values are ratios related to control with 95% confidence intervals of six separate experiments using different umbilical cords.

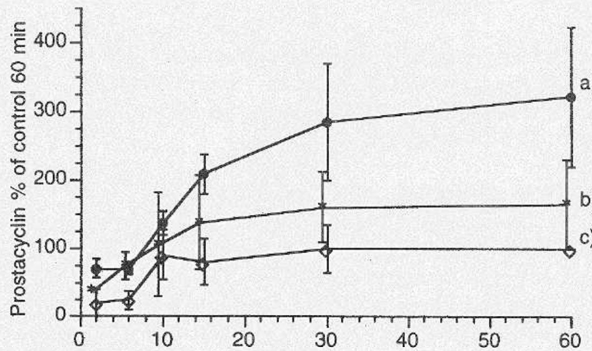


Fig 7. Time-course curves for PGI_2 production from human umbilical vein endothelial cells (HUVEC). Samples from the conditioned medium were collected after certain periods of time and analysed for the stable PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$. a) Cell dishes preincubated with metoprolol, 10^{-5} M. Isoprenalin, 10^{-6} M, added at zero time. b) Cell dishes incubated with isoprenalin alone, 10^{-6} M added at zero time. c) Control cell dishes exposed to cell medium only. The data were normalized to the results from control dishes at time 60 min =100%. Values are mean ± SD of 3-15 experiments.

Time-course

Time-course curves were constructed for the basal and stimulated PGI_2 production. After a short lag-phase of about 6 minutes, the concentrations started to increase rapidly. This phase was followed by a successively decreasing synthesis rate, with stable concentrations during the later part of the experiment. Values from samples drawn after 60 minutes represented the total PGI_2 production. The time-curves of the stimulated and the non-stimulated cells were different in shape. The stimulated cells demonstrated a prolonged phase of rapidly increasing concentrations. (Fig 7.)

t-PA and PAI-1 production

Basal production

The production of t-PA and PAI-1 varied between different cell cultures. Basal antigen production, presented as the mean basal antigen values and 95% confidence interval; n=numbers of cell dishes in the experiment.

In paper III, which examines human umbilical vein endothelial cells (HUVEC) and human adult vein endothelial cells (HAVEC), the basal antigen production was:

t-PA:

HUVEC 3,1±0.5 ng/10^5 cells, n=61,

HAVEC 2.2±0.6 ng/10^5 cells, n=24 and for

PAI-1:

HUVEC: 111±16 ng/10^5 cells, n=57,

HAVEC: 65±11 ng/10^5 cells, n=24.

In paper IV, which only examines HAVEC, the basal antigen production was:

t-PA: 1.5±0.3 ng/10^5 cells and for

PAI-1: 106±17 ng/10^5 cells, n=24.

Agonist-stimulated t-PA and PAI-1 production

All the data are expressed as percentages of basal t-PA or PAI-1 production (indexed to 100%) in the same experiment, mean ±95% confidence interval; n=numbers of cell dishes in the experiment.

Human umbilical vein endothelial cells (HUVEC)

In paper II, agonists for several different cell surface receptors and second messengers were used to explore the regulation of t-PA and PAI-1 production from human umbilical vein endothelial cells (HUVEC). Dose-response experiments were performed. Samples collected after 16 or 24 hours from the start of the experiment varied in a consistent way, which is why data from the 16-hour samples only are presented. Data are presented as peak or nadir values in Fig 8. For complete dose-response data see table I and II in paper II.

Forskolin (1uM - 500uM) induced a significant, dose-dependent decrease in PAI-1 production, at 100uM to 61%±16, n=12 of basal values. t-PA production was unaffected, (97%±16, n=13). These results were in contrast to the receptor agonists isoprenalin (10^{-9} M - 10^{-5} M) and ephedrine (10^{-8} M -10^{-4} M), which had no effect on either t-PA or PAI-1 production.

Na-nitroprusside (10^{-9} M - 10^{-5} M) induced an increased PAI-1 production, at 10^{-5} M to 141%±38, n=6, just reaching the level of significance. t-PA production remained

essentially unchanged.

Endothelin-1 (ET-1) (10^{-12} M - 10^{-8} M). The production of neither t-PA nor PAI-1 was significantly influenced by ET-1 at any concentration.

Phorbol 12-myristate 13-acetate (PMA) (1 ng/ml - 1 ug/ml =1.62nM - 16.2uM). PMA, in a dose-dependent fashion, significantly increased both t-PA and PAI-1 production. The mean PAI-1 production at 0.1ug/ml was 163%±35, n=20 and the mean t-PA was 350%±70, n=20, above basal control cultures.

Thrombin (10^{-3} U/ml - 10 U/ml). The production of t-PA and PAI-1 was increased significantly in response to thrombin at concentrations from 10^{-2} U/ml and up to 166%-195% and 130%-136%, n=10-17, above basal respectively.

Endotoxin (LPS) (10^{-3} ug/ml - 10 ug/ml). Significant increases were attained only for the highest LPS concentration (10ug/ml) with a mean t-PA of 130%±209, n=14 and PAI-1 production of 136%±30, n=15, above control values.

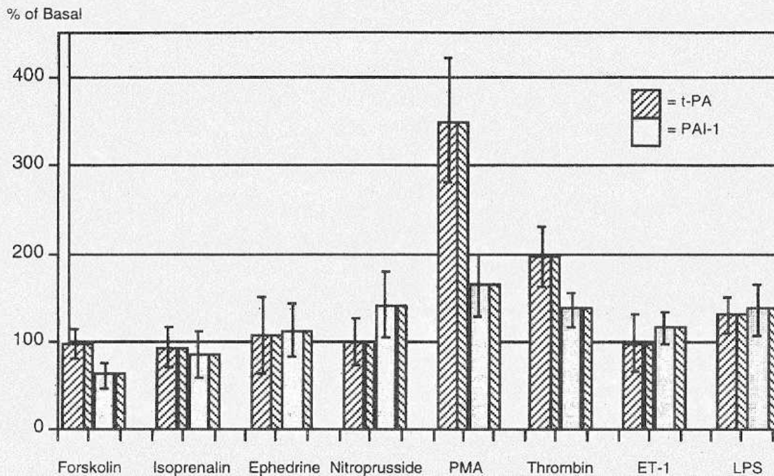


Fig 8. Peak and nadir values for tissue plasminogen activator (t-PA) and plasminogen activatot inhibitor type-1 (PAI-1) antigen from human umbilical vein endothelial cells (HUVEC). Data are expessed as a percentage of basal production (100%), means ±95% confidence intervals. Concentrations: forskolin 100uM; isoprenalin 10^{-5} M; ephedrine 10^{-4} M; Na-nitro-prusside 10^{-5} M; PMA (phorbol ester) 0.1ug/ml (=0.162uM); thrombin10 U/l; LPS (endotoxin) 10 ug/ml. Samples were collected after 16 hours´ of incubation.

Human adult vein endothelial cells (HAVEC)

In paper III, and in dose-finding experiments in preparation for this paper, the production of t-PA and PAI-1 from human adult vein endothelial cells (HAVEC) was examined. The cells were exposed to different agonists which were selected in accordance with the results in paper II. Samples were collected and analysed after 16 hours of exposure.

Forskolin (100uM) significantly decreased the basal PAI-1 production (42%±17, n=9) without affecting the basal t-PA production (90±18%, n=9). In experiments

presented in paper IV, a weak but significant reduction in t-PA production was demonstrated, (74%±10, n=6).

Phorbol 12-Myrstate 13-acetate (PMA) (0.1ng/ml =1,62uM) significantly increased the production of t-PA (1494%±407, n=22) and PAI-1 (221%±55, n=22).

Thrombin (10 U/ml) significantly increased the production of both t-PA (359%±70, n=9) and PAI-1 (231%±48, n=9). These results could be repeated in the experiments presented in paper IV.

Endotoxin (LPS) (10 ug/ml) had no significant effect on the t-PA (100%±20, n=16) or on PAI-1 production (107%±19, n=15) from HAVEC.

Modulation of agonist-stimulated t-PA and PAI-1 production "Cross-talk"

In paper III, the ability to modulate endothelial t-PA and PAI-1 production induced by thrombin, LPS or phorbolester, was examined. In the experiments, different combinations of the agonists and forskolin or the PKC-inhibitor H7 were performed and tested. The production of human umbilical (HUVEC) and human adult (HAVEC) endothelium was compared.

Co-incubation with H7

H7 (5uM - 100uM), a PKC inhibitor per se reduced PAI-1 production in HAVEC at 100uM (68%±17, n=15), but not significantly in HUVEC (82%±21, n=15). No effect could be demonstrated on t-PA production; HAVEC (96%±35, n=12), HUVEC (111%±17, n=14).

When exposed to PMA, HUVEC and HAVEC increased their t-PA and PAI-1 production. This induction was significantly reduced by co-incubating with the PKC inhibitor H7.

Thrombin stimulated both HUVEC and HAVEC to increase their t-PA and PAI-1 production. This agonist induction was significantly reduced by co-incubating with H7.

Endotoxin modestly increased the production of t-PA and PAI-1 from HUVEC. This effect could be inhibited by co-incubation with H7. LPS had no effect on adult cells. However, when combined with H7, their production of PAI-1 decreased below basal but their t-PA production was unchanged.

Co-incubation with forskolin

Forskolin, as a single drug, in these experiments significantly decreased PAI-1 production from both HUVEC and HAVEC without any effect on t-PA production.

In HAVEC, but not in HUVEC, forskolin acts synergistically with PMA or endotoxin, increasing t-PA production (by a factor of 1.9 and 1.6 respectively) (Fig 9,11).

Thrombin-induced t-PA production is modulated differently by forskolin depending on the type of cell. In HUVEC, the production was increased even further (by a factor of 1.3) but in HAVEC it was decreased (by a factor of 1.6) when thrombin was co-incubated with forskolin (Fig 10).

Examination of PAI-1 production revealed that, forskolin significantly decreased the response in HAVEC to PMA (by a factor of 2.7), to LPS (to 1.5), to thrombin (to 3.0).

Even in HUVEC, PAI-1 production was significantly decreased by co-incubating PMA (1.5), or LPS (1.6) with forskolin. However, in HAVEC, thrombin-induced PAI-1 production was unchanged when forskolin was added.

Fig 9 The production of **(t-PA)** antigen from human umbilical vein endothelial cells (HUVEC), and from human adult vein endothelial cells (HAVEC), after 16 hours´ incubation with phorbol ester 0.1 ug/ml (=0.162uM) and forskolin 100uM. Data are expessed as percentage of basal production (100%), means ±95% confidence intervals. Numbers of experiments: HUVEC: PMA n=20, PMA + forskolin n=12; HAVEC: PMA n=22, PMA + forskolin n=9.
* $p < 0.05$ vs. PMA.

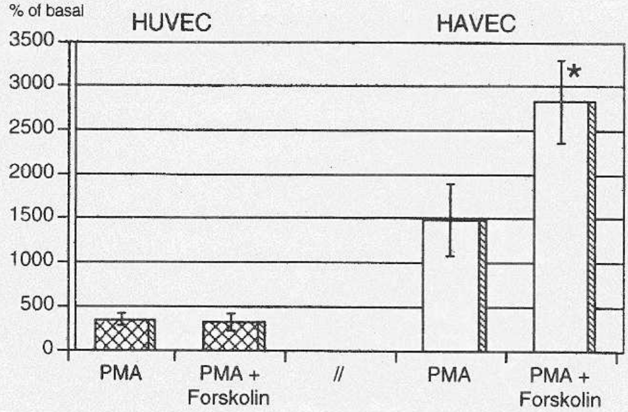


Fig 10 The production of **(t-PA)** antigen from human umbilical vein endothelial cells (HUVEC), and from human adult vein endothelial cells (HAVEC), after 16 hours´ incubation with thrombin 10U/ml and forskolin 100uM. Data are expessed as a percentage of basal production (100%), means ±95% confidence intervals. Numbers of experiments: HUVEC: thrombin n=20, thrombin+forskolin n=9; HAVEC: thrombin n=9, thrombin + forskolin n=9. * $p < 0.05$ vs. thrombin.

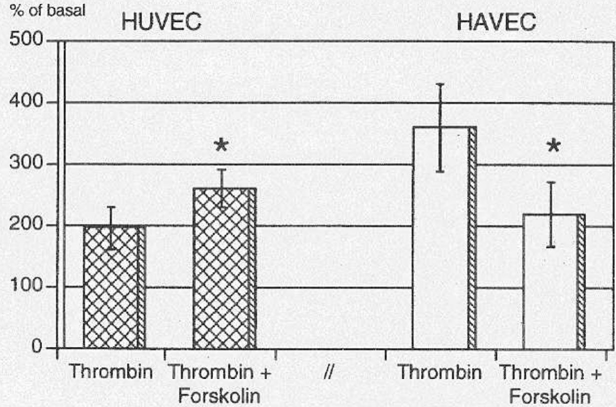
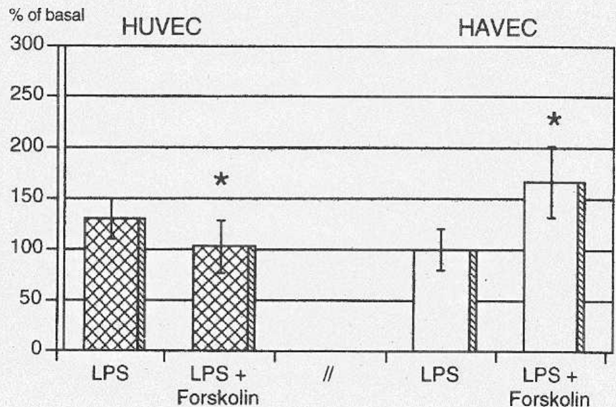


Fig 11 The production of **(t-PA)** antigen from human umbilical vein endothelial cells (HUVEC), and from human adult vein endothelial cells (HAVEC), after 16 hours´ incubation with endotoxin (LPS) 10ug/ml and forskolin 100uM. Data are expessed as a percentage of basal production (100%), means ±95% confidence intervals. Numbers of experiments: HUVEC: LPS n=14, LPS +forskolin n=9; HAVEC: LPS n=16, LPS + forskolin n=9. * $p < 0.05$ vs. LPS.



Thrombin signal transduction regulating t-PA and PAI-1 production

In paper IV, thrombin signal transduction preceding t-PA and PAI-1 production in adult endothelial cells, especially the role of $G_{\alpha i}$ -protein and tyrosine kinase, was examined. In preparation for this paper, a phosphatase inhibitor (sodium ortho-vanadate) was tested and comparative experiments were performed on human umbilical cells.

Pertussis toxin

In these first experiments, thrombin (10U/ml) significantly increased t-PA and PAI-1 production (234±42% and 211±42%, n=18 respectively). Pertussis toxin (100ng/ml), which inhibits $G_{\alpha i}$ -protein, reduced basal PAI-1 (66%±8, n=18) but only moderately affected basal t-PA production (88%±10, n=18). Cells pretreated with pertussis toxin produced significantly less t-PA and PAI-1 when stimulated with thrombin, (142%±23, n=18 and 146%±19, n=18 respectively). Forskolin, (100uM) in this experiment, had a weak influence on basal t-PA production (74%±10, n=6) but a stronger effect on basal PAI-1 production (51%±4, n=6) of non-stimulated value. Forskolin could significantly decrease thrombin-induced production of both t-PA and PAI-1 (91%±7 and 46%±8, n=6, respectively). When pertussis toxin was also added to the co-incubation between thrombin and forskolin, only a very limited additional inhibitory effect could be recorded. t-PA production decreased (75%±15, n=6) and PAI-1 was virtually unchanged (50%±5, n=6) (Fig 12).

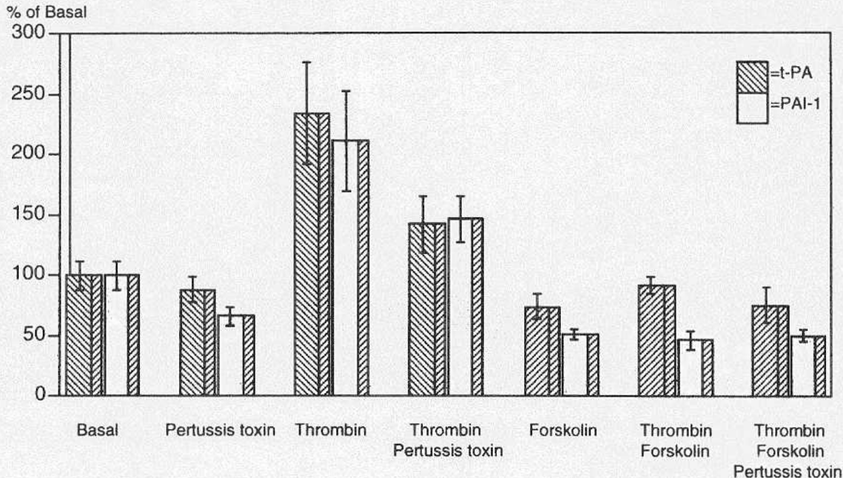


Fig 12. The production of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1) antigen from human adult vein endothelial cells (HAVEC), after 16 hours´ incubation with thrombin 10U/ml, n=18; pertussis toxin (PTX) 100ng/ml, n=18; forskolin 100uM, n=6, and combinations (thrombin + forskolin n=18 ; other combinations, n=6. Data are expressed as a percentage of basal production (100%), means ±95% confidence intervals.

Genistein, Sodium ortho-vanadate

Genistein, a tyrosine kinase inhibitor, was tested in a separate set of experiments against thrombin. Preincubation with genistein significantly reduced thrombin induction of both t-PA and PAI-1 production. The effect was dose-dependent. In these experiments, genistein, 2.5ug/ml or 10ug/ml or 40ug/ml, reduced the thrombin-stimulated t-PA production from 315%±89 to 128%±54, 98%±18 or 77%±14, n=6 respectively. The thrombin-stimulated PAI-1 was decreased from 282%±96 to 78%±35, 32%±9 or 29%±17, n=6 respectively.

Sodium ortho-vanadate, a tyrosine phosphatase inhibitor per se (30uM), had no significant influence on either basal t-PA (140%±57) or PAI-1 (94%±31, n=6), production. When combined, vanadate 30uM or 100uM reduced thrombin-stimulated t-PA production in this experiment from 310%±45 to 204%±69 or 232%±53, n=6, and thrombin-stimulated PAI-1 from 248%±38 to 129%±39 or 123%±29, n=6. (Fig 13).

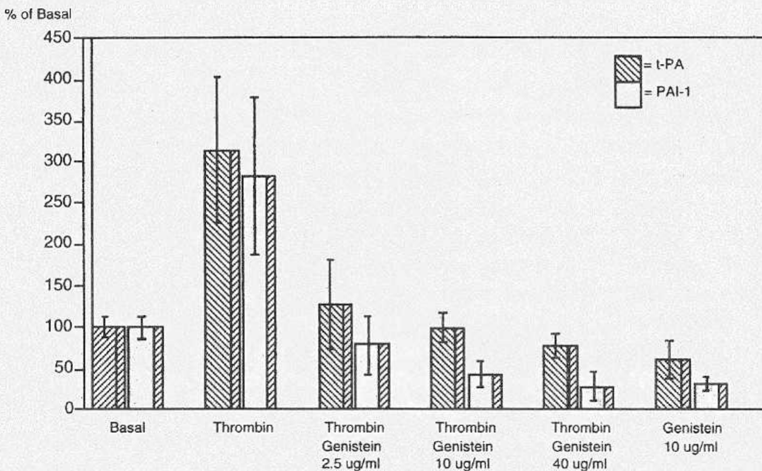


Fig 13. The production of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1) antigen from human adult vein endothelial cells (HAVEC), after 16 hours´ incubation with thrombin 10U/ml, genistein 2.5 - 40ug/ml, and combinations for 16 hours, n=6. Data are expressed as a percentage of basal production (100%), means ±95% confidence intervals.

DISCUSSION

Discussion of methods

A cell culture system allows comparisons to be made under defined and identical experimental conditions. Even if the use of culture systems has been almost routine in metabolic studies (Kooistra 1990), it is important to consider that the extrapolation of data to the in-vivo situation has to be carried out with great care.

Methods in prostacyclin study

Endothelial cells in culture, have been demonstrated to be a suitable model for functional pharmacological studies exploring prostacyclin production. When synthesized, prostacyclin is instantly released from the cell and no intracellular storage pools have been detected (Levin 1984, Jaffe 1987, Dusting 1990, Gerritsen 1996). Hence accumulation in the conditioned medium is likely to represent the total PGI_2 production.

Prostacyclin (PGI_2) has a short half-life and spontaneously converts to the inactive metabolite 6-keto $PGF_{1\alpha}$ (Moncada 1990, Dusting 1990). In vitro, antigen measurement of this metabolite is representative of PGI_2. However, in-vivo plasma measurements of 6-keto $PGF_{1\alpha}$ are an unreliable method and the analysis of urine excretion of 6-dinor-keto- $PGF_{1\alpha}$ has proved to be a better one (Greén 1986, Wennmalm 1990, 1992). The importance of having a specific antibody has been stressed (Patrono 1987). The antibody used in this experiment is rather selective and the method reliable in the actual concentrations.

Cyclooxygenase being an irreversible desensitizating enzyme complex needs a lag-phase of protein synthesis to restore enzyme activity after inactivation (Duniec 1990, Shimizu 1994, Gerritsen 1996). Consequently, the pre-experimental handling of the cells is crucial for the PGI_2 production. In the present study, the cell cultures have been investigated, unperturburated at confluence.

Cells cultures originating from different umbilical cords have been reported to have large quantitative but no qualitative differences in PGI_2 production (Adler 1981, Levin 1984). To correct for these variations, all the data were related to the basal non-stimulated cultures in the same experiment.

Concentration of β-adrenoceptor blocker metoprolol and propranolol (10^{-5} M) were selected to give a high receptor binding rate. The peak plasma concentrations of metoprolol in vivo reaching 10^{-6} M (Harron 1981, Sandberg 1988). At a plasma-correlated metoprolol concentration of 10^{-5} M, the β-adrenoceptor blockade was maximal for S-isomers but only about 15% for R-isomers (Wahlund 1990).

Methods in tPA PAI studies

Endothelial cells in culture constitute a well-documented experimental model for studying tPA and PAI-1 production (Gerard 1989, Loskutoff 1989, Kooistra 1990, 1994). For t-PA and PAI-1, correlations have been demonstrated between the following methods: immunohistology, in situ hybridization, tissue extraction analysis and protein secretion (Schleef 1990, Quax 1990, Simpson 1991, Sawdey 1991, Keeton 1993, vd Eijnden-Schrauwen 1995, Padró 1990). The production of t-PA or PAI-1 from cultured cells could be estimated by measuring activity or antigen in the conditioned medium or by quantifying intracellular mRNA. Antigen and mRNA methods are time specific but comparable for the detection of total t-PA or PAI-1

production, (Declerch 1988, Sawdey 1991, Arnman 1993), whereas activity methods measure the sum of activators and inhibitors (Eriksson 1988). As different antibodies are sensitive for specific epitopes or molecular conformations of t-PA and PAI-1, absolute values using different antibodies could not be compared (Perrie 1993). In the present studies, a polyclonal coating-antibody, recognising all forms of t-PA or PAI-1, was used. Measurements were performed at reliable antigen concentrations.

The acute release of t-PA from cellular pools and of PAI-1 from extra-cellular matrix pools have been described. These pools were quite small and the release correlated to the slower cellular synthesis rate (Levin 1987, Knudsen 1988, Mimuro 1989a, Schleef 1990, vd Eijnden-Schrauwen 1995). Hence the antigen accumulation in the medium during the experiment was likely to represent the total cellular t-PA and PAI-1 synthesis (Schleef 1990, Kooistra 1994, vd Eijnden-Schrauwen 1995).

The synthesis of t-PA and PAI-1 from cultured endothelial cells could be influenced by several factors: phenotype, number of passages, state of confluence or culture medium content. Even under identical culture conditions, large quantitative variations have been observed between different cultures (v Hinsbergh 1987, Dichek 1989). To reduce these problems in the present study, both cellular and cultivation conditions were kept identical in all the experiments. Absolute production data were related to basal, non-stimulated, control cultures in the same experiment.

Serum, added to the culture medium, contains several different stimulating substances such as growth factors including TGFβ (v Hinsbergh 1987, Cobb 1991, Moolenaar 1995). To minimize this non-specific effect, which interferes with the production of t-PA and PAI-1, the experiments were performed under serum-free conditions. The release of t-PA and PAI-1 to the conditioned medium, after a lag-phase, progressed at a relatively constant rate. The duration of the incubation has to be adjusted for the sensitivity of the measuring methods, the pharmacology of the test substances and for practical reasons. A period of 16 hour´ exposure to the test drugs was selected in the present study as the optimal experimental time.

Prostacyclin production

Prostacyclin, secreted from endothelial cells, could decrease platelets aggregation and adhesions but could also relax smooth muscle cells in the vessel wall (Moncada 1990, Vane 1990, Busse 1993).

Prostacyclin, Hypertension, Adrenergic tonus

In a population with normal blood pressure, a relationship has been demonstrated between high adrenergic tonus and an increased production of PGI_2 (Wennmalm 1990). On the other hand, various studies have demonstrated that individuals with hypertension have high adrenergic tonus (Tuck 1986, Åblad 1988) and paradoxically low PGI_2 production (Fitzgerald 1987, Beckmann 1988, Frölich 1990, Minuz 1990).

A hypothesis is that decreased prostacyclin production resulting from a patho-physiological adaptation to high adrenergic tonus could be of etiologic importance in hypertension. The adrenergic modulation of endothelial prostacyclin production has attracted great interest (Adler 1981, Pettersson 1991), but is still not completely understood. We have tested the hypothesis that prostacyclin production is regulated by adrenergic mechanisms.

Results in the present study

In our pre-non-stimulated cell-culture model, the β-adrenoceptor agonist

isoprenalin gave a small but significant increase in PGI_2 production. This result corroborated other in-vivo studies (Schaffer 1982, Williams 1989, Wennmalm 1990) but diverged from certain in-vitro studies (Adler 1981, Jeremy 1985). However, Adler and Jeremy used pre-stimulated cell models, constructed to estimate the capacity of isoprenalin to modulate an ongoing PGI_2 synthesis and not to initiate the synthesis.

In the present study, a combination of isoprenalin and a β-adrenoceptor antagonist, metoprolol or propranolol, increased the PGI_2 production even further. This synergism has not previously been reported when cultured endothelial cells have been used. However, similar effects have been demonstrated in other experimental systems, in vivo from humans (Beckmann 1988) and rabbits (Åblad 1988) and in vitro on guinea pig hearts (Löbel 1985) and rat aortas (Nishimiya 1990, Hirawa 1991).

The possibility that PGI_2 was increased by β-adrenoceptor blockers alone could be contradicted, as in the present study, metoprolol or propranolol did not significantly affect the basal PGI_2 production. This result agreed with other reports in vivo (Greén 1986, Wennmalm 1992) and in vitro (Adler 1981, Nishimiya 1990, Hirawa 1991).

To further explore the synergistic effect of isoprenalin and β-adrenoceptor blockers, metoprolol isomers with either high or low receptor blocking activity were examined. Both isomers, when co-incubated with isoprenalin, were found to have an equally synergistic effect on PGI_2 production. Consequently, the synergistic effect could be separated from the β-adrenoceptor antagonistic effect of metoprolol. Effects not related to the β-adrenoceptor blocking capacity have been reported earlier for different isomers of propranolol (Cambell 1981, Chobanian 1985, Callahan 1986). However, other studies have demonstrated effects caused only by the active isomer (Löbel 1985, Nishimiya 1990).

Intracellular cAMP, induced by adrenoagonists, has been reported to have a short half-life due to phosphodiesterases (PDE) activity. To strengthen any cAMP effect in the experiment, we and others have inhibited PDE with isobutylmetylxantin (IBMX), a well-documented inhibitor of different PDE isomers (Adler 1981, Hall 1989, Manolopoulos 1995a). IBMX has also been demonstrated to have other effects, inhibiting adenosine receptors (Fredholm 1980) and, in high concentrations, even phospholipase C (PLC) (Matsuzawa 1980). Brotherton (Brotherton 1982) pointed out that IBMX could have an inhibitory effect on PGI_2 production in experimental cell models when pre-PLC-stimulation was used. In our experimental cell model, no pre-PLC-stimulation or adenosine agonists that could be inhibited by IBMX were used. Hence in the present study, IBMX per se had no effect on basal PGI_2 production.

Signal transduction - Hypothesis

These experiments could not clarify the intracellular interactions behind the synergistic effect of isoprenalin and metoprolol or propranolol. However, from studies using other experimental cell systems which explored signal transduction, a working hypothesis could be formulated. The hypothesis will be discussed in the following section, structured according to the site of interaction: 1) with phosholipase A_2 (PLA_2), the key enzyme in the cascade, 2) with the β-adrenoceptor or 3) with other pharmacological side effects.

1) Interaction with PLA_2

Recent reports have described two mechanisms responsible for PLA_2 activation.

a) Ca^{++} induces translocation of cytosolic PLA_2 to the membrane creating direct contact with the phospholipid substrates.

b) MAP kinase phosphorylation of PLA_2 increases the activity of the enzyme (Lin 1993).

a) Ca^{++} induced translocation of cytosolic PLA_2

There are, in principle, two ways to increase the intracellular Ca^{++} concentration: a rapid receptor-regulated release from intracellular storages and a slower trans-membrane influx (see Introduction).

The release from intracellular stores (ER) is dependent on receptor-mediated PLC/ IP_3 activity and no β-adrenergic regulation has been described.

The transmembrane Ca^{++} influx could be regulated both by a controlled opening of the transmembrane ion-channels and by the electrical ion-force created by the membrane potential. In endothelial cells, which have no voltage-dependent channels (Adams 1989), the probability of a Ca^{++} ion-channel opening is regulated by receptors (Lückhoff 1992). Membrane hyperpolarization by K^+ influx, increases Ca^{++} ion force and influx. In platelets and cardiac muscle cells, adrenoceptors connected to GTP-binding proteins could regulate K^+ channels and the Na^{++}/H^+ pump (Sweatt 1986, Barber 1989, Schubert 1989). In endothelium, K^+ channels are regulated by cell receptors, GTP-binding proteins, cAMP, intracellular Ca^{++} and H^+ concentrations (Adams 1989, Siegel 1991). Sheer stress could also open endothelial K^+ channels (Olesen 1988), and activate tyrosine kinases (Berk 1995) corresponding to the flow-induced PGI_2 production (Frangos 1985). Increased intracellular pH, created by the Na^{++}/H^+ antiport (Madshus 1988), could increase PLA_2 activity, both in platelets (Sweatt 1986, Limbird 1988) and in endothelium (Kitazono 1989).

The cytosolic Ca^{++} concentration could be decreased by active ion-pumps. cAMP / PKA modulation of these pumps has been described for smooth muscle and cardiac muscle cells but not for endothelium (Braunwall 1982).

From this section (1a), the following hypothesis may be formulated:

Isoprenalin and/or metoprolol / propranolol could (via GTP-binding proteins) induce a shift in transmembrane K^+, Ca^{++}, H^+- flux. These shifts could increase, directly or indirectly, intracellular Ca^{++} or pH, stimulating PLA_2 activity and PGI_2 production.

b) MAP kinase phosphorylation of PLA_2

MAP kinase activity has proved to be a converging point for several signals induced by GTP-binding proteins, growth factors, cytokins, adhesion molecules and sheer stress (Johnson 1994, Seger 1995, Post 1996). Since MAP kinase could phosphorylate and activate PLA_2 (Lin 1993). Tentatively, interference with preceding enzyme-steps in the MAP cascade could modulate the final PGI_2 production.

Both isoprenalin, through the β-adrenoceptor (Barber 1989, Schubert 1989, Williams 1989, Yatani 1989, Williams 1995) and receptors linked to the PLC system (Burch 1990, Garcia 1992), has been reported to dissociate GTP-binding proteins that could activate PLA_2. Ras, an oncogen preceding MAP-kinase, could be inhibited by cAMP/PKA (Johnson 1994). This might be the point of action for cAMP/PKA-inhibited PLA_2 reported in platelets (Schafer 1984). However, endothelial data

according this effect diverge (Adler 1981, Brotherton 1982, Hong 1985).

From this section (1b), the following hypothesis may be formulated:

a) Isoprenalin could stimulate, directly or indirectly, the MAP-kinase cascade and further PLA_2 by dissociating membrane GTP-binding proteins (Schubert 1989, Garcia 1991, Clark 1993, Post 1996).

b) The MAP kinase cascade could be inhibited at the Ras/raf-1 step by cAMP / PKA (Johnson 1994, Post 1996). Metoprolol/ propranolol could either reduce the cAMP/PKA inhibition or increase the MAP-kinase stimulation, creating a synergistic effect on PGI_2 production.

2) Interactions with the β-adrenoceptor

The β-adrenoceptor could be rapidly and reversibly desensitizated by PKA. This inhibition could be further increased by β-AR kinase, phosphorylating the receptor. After prolonged agonist exposure, the receptor degrades and has to be replaced by a new protein synthesis (Hausdorff 1990, Keen 1992). In perfused rabbit hearts, Williams (Williams 1989) found isoprenalin-induced prostacyclin production to be paradoxically decreased by cAMP/PKA. The results indicated a non-cAMP-transducted prostacyclin-increasing effect of $G_{\alpha s}$, as described for ion-channels (Barber 1989, Schubert 1989, Yatani 1989, Williams 1995). Furthermore the effect of β-AR kinase could be increased and the β-adrenoceptor desensitized by PKC (Winsel 1996). Even other pathways, phosphorylation β-AR kinase, probably exist, but the regulation is still unknown. By rearranging membrane structures PLA_2 activity could also decrease the receptor binding capacity (Cohen 1985, Williams 1995).

3) Interactions with other pharmacological side effects

By sensitizing PLA_2 for Ca^{++}, calmodulin has been demonstrated to increase the activity of PLA_2 in platelet, hepatic and gastric mucosal cells (Vanderhoek 1979, Matzusawa 1980, Olaisson 1990), although contradictory results have been published on endothelial cells (Adler 1981, Jaffe 1986). Propranolol in high doses has been reported to inhibit calmodulin, an effect not related to the β-adrenoceptor (Volpi 1981).

Propranolol in high doses has also been demonstrated to inhibit platelets PLA_2, (Vanderhoek 1979, Cambell 1981, Callahan 1985), hepatic phospholipase C (PLC) (Matzusawa 1980), proteinkinase C (PKC) and the conversion of phosphatic acid (PA) into diacylglycerol (DAG) / arachidonic acid (Sozzani 1992). These effects were not related to the β-adrenoceptor. On the other hand, Das (Das 1988), using human neutrophils, demonstrated increased inositol-phosphate production (PLC effect) when propranolol was added.

Cyclooxygenase is irreversibly auto-desensitized when converting arachidonic acid to prostacyclin precursors (Shimizu 1994, Gerritsen 1996). This desensitization could be prevented by scavengers. Löbel (Löbel 1984) hypothesized that β-adrenoceptor blockers could act as scavengers.

From this section (3), which mainly reports inhibitory side-effects of propranolol, no hypothesis could be formulated to fit the stimulatory effect that was found in the present study. The results reported by Das and Löbel could have been of interest, but they have not been confirmed by others.

Summary

In the present study, isoprenalin and β-adrenoceptor antagonists such as metoprolol or propranolol synergistically increased prostacyclin production from endothelial cells. The effect could not be associated with the β-adrenoceptor-blocking effect per se. The intracellular background for this synergism is not clear at present, although a working hypothesis could be formulated.

The results corroborated previous clinical reports and may offer a hypothesis as to why only hypertensive individuals, characterised by high basal adrenergic tonus and paradoxically low PGI_2 production, react with increased PGI_2 production when undergoing β-adrenoceptor-blocking therapy.

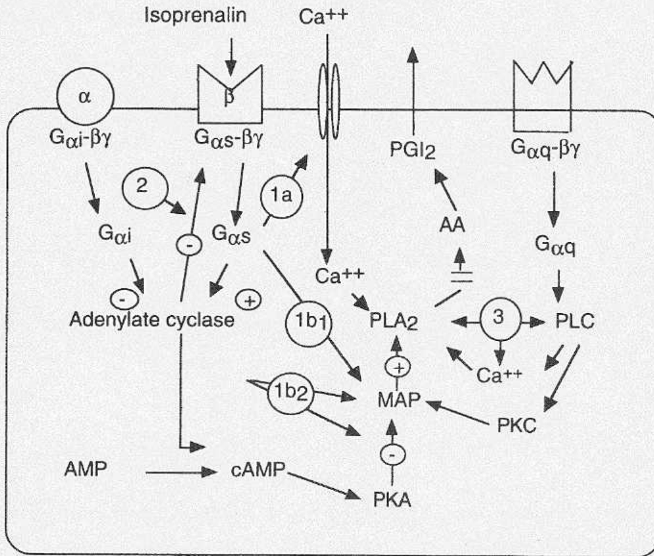


Fig 14. Intracellular interactions behind the synergistic effect of isoprenalin and β-adrenoceptor antagonists, on endothelial prostacyclin production. Summary of hypothesis.

t-PA and PAI-1 production

Agonist signals could be transducted through many different "cross talking" intracellular pathways. To explore these interactions and to modulate endothelial t-PA and PAI-1 production, substances with well-known intracellular pathways were tested. Furthermore, endotoxin, a substance with high biological activity but unclassified signal transduction, was selected. In a first series of experiments (paper II), the substances were added as single drugs and dose-response curves were performed. With the results in paper II as a basis, phorbolester, thrombin and endotoxin were selected to be co-incubated with forskolin or the PKC inhibitor, H7 (paper III). Thrombin, being a central biological substance with complex trans-duction, was further examined using inhibitors to GTP-binding proteins and MAP kinases (paper IV). The structure in the following section is based on a presentation of examined agonists, with or without co-incubation. Agonists using the same signal pathway have been presented together.

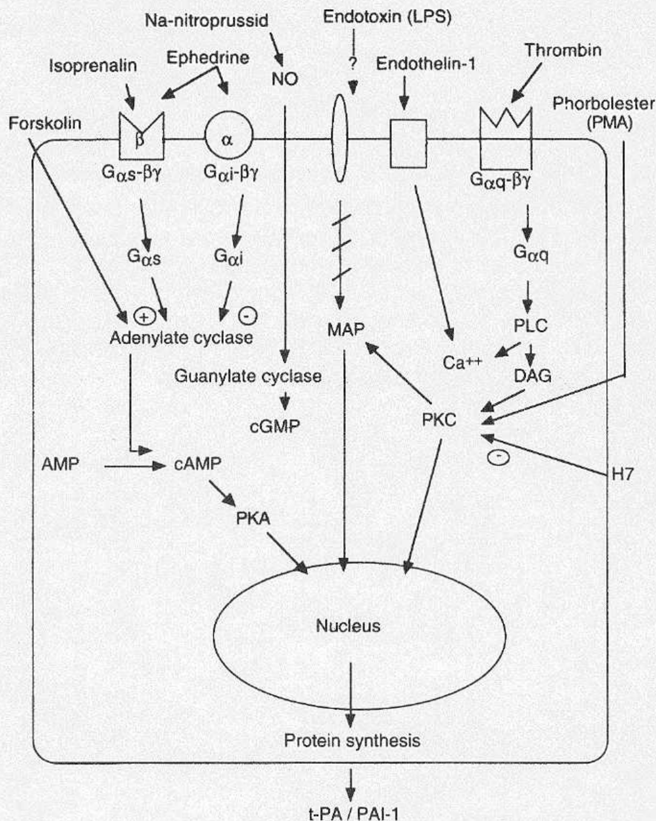


Fig 15. Agonist stimulated t-PA and PAI-1 production. Summary of explored pathways.

Isoprenalin, with predominating β- and **ephedrine** with combined α- and β-adrenoceptor activation. The β-receptor is connected to the $G_{\alpha s}$ -protein and the α-receptor to the $G_{\alpha i}$ -protein. The activity of the enzyme, adenylate cyclase, producing cAMP, could be increased by $G_{\alpha s}$ or decreased by $G_{\alpha i}$ respectively (Sibley 1985, Limbird 1988). In the present study (paper II), using umbilical cells, neither isoprenalin nor ephedrine demonstrated any influence on t-PA or PAI-1 production.

Forskolin binds to and directly activates adenylate cyclase, increasing the intracellular level of cAMP (Sibley 1985, Iyengar 1993, Manolopoulos 1995a). In the present study (papers II, III), both adult and umbilical cells exposed to forskolin significantly decreased PAI-1 production but had only minimal influence on t-PA production. These results were in line with other reports (Santell 1988, Francis 1989, Kooistra 1991, Slivka 1991). The divergence in cAMP-modulated production indicated separate regulatory pathways for t-PA and PAI-1. The incapacity of isoprenalin to influence PAI-1 could be explained by rapid adrenoceptor desensitization following agonist exposure. Forskolin with a direct enzyme activation engendered no desensitization (Sibley 1985, Hausdorff 1990).

Na-nitroprusside (NP) has been demonstrated to generate nitric oxide (NO), stimulating guanylate cyclase to produce cGMP (Brotherton 1986, Harrison 1993).

In the present study (paper II) in which umbilical cells were used, NP increased PAI-1 production slightly, albeit significantly, but had no influence on t-PA production. However, the results could be disputed as the number of experiments was low and the effect not strictly dose-dependent. No NP effect could be detected on the rapid t-PA release from rat and human cells respectively (Tranquille 1993, Jern 1994). Increased fibrinolysis has been reported in rabbit; following NP administration, the hypothesis being that this is due to an inhibitory effect on PAI-1 release from platelets (Lidbury 1990). In-vitro cGMP was described (data were not presented) as having no influence upon PAI-1 incorporation in matrix (Slivka 1991). In a recently published study, no influence on either t-PA or PAI-1 production could be demonstrated when umbilical cells incubated with NP and fetal calf serum were used (Pannocchia 1996).

Endothelin-1 (ET-1) has been demonstrated to be a potent vasoactive polypeptide in the endothelin family. ET-1 could activate a set of different endothelin receptors, inducing biological effects on several types of cell. As they have non-selective ET_{B1} receptors, endothelial cells could, in a autocoid manner, both produce and respond to ET-1. The cell receptors belong to the seven transmembrane-spanning, and GTP-binding-protein family. In endothelium, the receptor has been reported to be connected to $G_{\alpha q}$ and $G_{\alpha i}$-protein. Agonist-binding increased cytosolic Ca^{++} concentration by activation of phospholipase C and by opening cell membrane channels. In endothelium, ET-1 releases NO and PGI_2 (Vane 1990, Masaki 1991). ET-1 could also decrease cAMP (Eguchi 1993).

In the present study (paper II) on umbilical cells, incubation to ET-1 had no effect on their t-PA or PAI-1 production. Results from other studies exploring the effect of endothelin on fibrinolysis diverge. In a study using rabbits, administration of ET-3 increased fibrinolysis (Kornbut 1989). In an in-vitro study, an umbilical cell-line, when exposed to ET-1, decreased t-PA production 25%, but had no effect upon PAI-1 production (Yamamoto 1992).

Protein kinase C (PKC) has revealed itself to be a potent intracellular phosphorylating enzyme, central to signal transduction. PKC has been demonstrated to modulate receptors, GTP-binding proteins, membrane lipases, ion-channels, intracellular enzymes and transcription of both pro-oncogenes and secreted proteins. The multi-substrate capacity of PKC could result in a broad spectrum of diverging intracellular signals. The final cellular response upon PKC activation is determined both by the intracellular localisation and by other modulating transducted signals (Kooistra 1991, Kolch 1993, Lin 1993, Nishizuka 1995, Thanos 1995, Winstell 1996).

Diacylglycerol (DAG), a metabolite with a short half-life produced by membrane phospholipase C or D is the natural activator of PKC. The effect of DAG could be imitated by exogenously-added phorbolester (PMA), producing long-lasting PKC activation. When activated, cytosolic PKC translocates and binds to the cell membrane. PKC exists in the form of several different isomers, which have different characteristics (Nishizuka 1995). In the present study, PMA increased t-PA and PAI-1 production, both in umbilical and adult endothelial cells. Adult cells are proportionally even more active in respect of t-PA production. Several studies have presented similar results (Santell 1988, Levin 1989, Francis 1989, Slivka 1991, Kooistra 1991).

H7, an isoquinoline sulfonamide, has been presented as a dose-dependent semi-selective PKC inhibitor. A high concentration of H7 could also inhibit cAMP (PKA) and

cGMP-dependent protein kinase (Hidaka 1987). In our experimental model, both H7 and forskolin decreased PAI-1 production, but only forskolin increases PKA activity. It thus seemed unlikely that H7 had any significant PKA-inhibiting effect. As cGMP has been demonstrated to have a minimal effect on fibrinolysis (paper II), a potential inhibiting effect by H7 must be insignificant.

In the present study, adult endothelial cells exposed to H7 decreased PAI-1 production significantly, whereas production in umbilical cells was insignificantly reduced. However, t-PA production was not influenced in any type of cell (paper III). In control experiments, both adult and umbilical cells reduced PMA-induced t-PA and PAI-1 production when co-incubated with the PKC inhibitor H7. The reduction was approximately 50% at a H7 concentration of 100uM (paper III). Similar results have been reported earlier (Levin 1989, Grulich-Henn 1990, Shi 1996).

In paper III, we examined the production of t-PA and PAI-1 from cells exposed to a combination of PMA and forskolin. In both types of cell, PAI-1 production was found to be decreased by co-incubation with forskolin compared with cells exposed to PMA alone. The reduction was proportional to the effect of forskolin per se, contradicting synergism. Production of t-PA induced by PMA was further increased by co-incubation with forskolin in adult, but not in umbilical, cells.

Several reports demonstrated that forskolin, or other cAMP-increasing substances, could decrease PMA-induced PAI-1 production (Santell 1988, Slivka 1991, Kooistra 1991). On the other hand, when umbilical cells were used, the lack of synergism in t-PA production was at variance with some of these studies (Santell 1988, Kooistra 1991). However, the experimental models were not identical with respect to serum and growth factor supplements.

Construction of the t-PA gene with several dynamic, positive and negative modulatory feedback mechanisms might explain both the synergistic effect and the diverging results in different reports (Medcalf 1990, Ohlsson 1992, Karin 1992). The human t-PA promotor contains a TATA box, but the most important points of regulation have been demonstrated to be two GC-boxes further downstream. The first GC box harbours a "CRE-like" sequence with one nucleotide shift inside and several changes outside the CRE-sequence, giving a low affinity for CREB. This sequence could bind the PKC-induced jun/fos dimers from the AP-1 family instead (Ohlsson 1992). The other GC-box harbours an AP-2 response element that could be activated both by CREB/ ATF and jun/fos dimers. The "CRE-like" and AP-2 -responding elements could act separately or synergistically when stimulated with PMA-activated PKC /jun/fos and forskolin-induced cAMP/CREB (Rickles 1989, Medcalf 1990). Jun/fos and CREB/ATF are simultaneously both activated and inhibited by transcription factors whereby TF´s creates a unique and dynamic regulatory balance (Karin 1992).

The human PAI-1 promotor regulates through a specific set of protein - nucleic acid interactions, but with a higher degree of conservation between species than the t-PA promotor (Loskutoff 1991). In addition to jun/fos, even c-myc, a transcription factor activated by the tyrosine kinase cascade, has been reported to regulate the PAI-1 gene (Prendergast 1990).

Endotoxin (lipopolysaccharide - LPS) a component of the outer wall of Gram-negative bacteria. LPS has been reported to have extensive biological effects, including increased production of PAI-1 in vivo (Colucci 1985, Sawdey 1991). The sensitivity reported for LPS varies remarkably between different experimental models. In vivo, LPS had an effect in the pg/ml range but, in vitro, when endothelial cells were used, the concentration had to be in the ug/ml range to be active. The in-vivo concentrations correspond to the LPS sensitivity of cytokin-producing mono-

cytes (Müller 1993, Redl 1993). Consequently, the LPS effect in vivo on endothelium are probably indirect, and due to cytokin-producing immune-competent cells (Loskutoff 1991, Kruithof 1993). t-PA production has been reported to be decreased by LPS (Kooistra 1994, v Hinsbergh 1994). Cellular transduction of the LPS signal still remains to be clarified. Endotoxin has been reported to induce intracellular phosphorylation, PKC and Nuclear Factor-kappa-B activation (Nakano 1993, Hill 1995, Thanos 1995). Several surface receptors have been proposed. Some of the receptors preferred to bind LPS in complex with the acute phase LPS-binding protein (Morrisson 1993). Serum requirement has also been reported in vitro and are probably a reason for low LPS reactivity in our experimental model (vd Berg 1988, Sawdey 1989).

In the present study, endotoxin, in a serum-free medium, had no influence on the production of t-PA and PAI-1 from adult endothelium. However umbilical cells just reached the level of significantly increased t-PA and PAI-1 production, when the highest LPS concentration (10ug/ml) was added (papers II, III). LPS-increased t-PA and PAI-1 production were reduced when co-incubated with the PKC inhibitor H7 (paper III). Similar results has been demonstrated by Nakano (Nakano 1993). Co-incubating LPS with forskolin decreased PAI-1 production in both adult and umbilical cell. This data was in line with other reports, but might even be a direct non-LPS influenced effect of cAMP (Slivka 1991, Ollivier 1993). The effect of forskolin on LPS increased t-PA production diverged between the two types of cell, adult cells further increased and umbilical cell decreased their production (paper III). Taken together, these results indicated a PKC-dependent LPS pathway with cellorigin specific cAMP modulation. However, the reliability of these data when extrapolated to the in-vivo situation could be discussed.

Thrombin the final enzyme in the coagulation cascade induces fibrin formation and pursues both positive and negative feedback on different coagulation factors. Further thrombin could enhance platelets activation and aggregation. Thrombin has also been demonstrated to cleave a short peptide from the N-terminus of a specific thrombin cell-surface receptor. The peptide could bind to and activate the receptor. When activated, the receptor molecule changes conformation, unmasking new intra-cellular binding sites. The newly discovered receptor, belonging to the seven trans-membrane-spanning receptor family, transducts signals through the GTP-binding protein system. When cleaved and activated, the receptor has to be re-placed and a state of desensitization occurs. Endothelium, in contrast to platelets, has a pool of new receptors that could be recruited from intracellular vesicles. The receptor-binding capacity could also be modified by phosphorylation, and in analogy with the β-adrenoceptor even protein kinase A could interact (v Obberghen-Schilling 1993, Wilhelm 1993, Brass 1995, Storck 1996). Following vascular injury, receptor expression is increased in vessel-wall smooth muscle cells, neointima (Runge 1995).

In endothelium, thrombin signals have originally being described as transduced through the $G_{\alpha q}$-protein pathway, which, in turn activates phospholipase C (PLC), diacylglycerol (DAG), Ca^{++} and protein kinase C (PKC) (Garcia 1992, Brass 1995). Recent research has demonstrated that, depending on the type of cell, the thrombin signal could dissociate several different types of G_{α}-proteins from their $\beta\gamma$-dimers. In endothelial cells, $G_{\alpha i}$-protein has been reported to be activated by thrombin. Hypothetically, the $G_{\alpha i}$ / $\beta\gamma$-dimer pathway can influence adenylate cyclase, and the mitogen-activated protein (MAP) kinase cascade (Garcia 1992, v Obberghen-

Schilling 1993, Brass 1995). In some types of cell, $\beta\gamma$-dimers have been found to increase the activity of specific PLC-isomers (Birnbauer 1992, Dickenson 1993). However, in human endothelium pertussis-toxin, inhibiting the $G_{\alpha i}$-pathway had no effect on thrombin-induced PLC activity, indicating the presence of $\beta\gamma$-dimer - insensitive PLC-isomers (Garcia 1992).

In the present study, thrombin increased the production of t-PA and PAI-1 both from umbilical and adult cells, the latter being the most reactive (papers II-IV). This results were in line with other studies both in endothelium (Gelehrter 1986, Dichek 1989, Grulich-Henn 1990). The PKC inhibitor H7 counteracted thrombin-increased production of t-PA and PAI-1, indicating the existence of the thrombin G_{aq} / PKC pathway (paper III), which corroborated data by others (Grulich-Henn 1990). When co-incubated with forskolin, adult cells reduced thrombin induction of t-PA and PAI-1 production. These data were in line with a previous report (Francis 1989). On the other hand, umbilical cells demonstrated increased t-PA and unchanged PAI-1 production. It has not been possible to rule out the mechanisms producing this diverging modulator effect of forskolin on adult and umbilical cells. A tentative theory is that different preferential pathways ($G_{\alpha i}$ / $G_{\alpha q}$) or a shift in the balance between stimulatory and inhibitory feedback mechanisms might be conceivable.

Pertussis toxin (PTX) as a selective inhibitor of the $G_{\alpha i}$ sub-group of GTP-binding proteins has became a frequently used tool in transduction research (Hepler 1992).

In the present study (paper IV), PTX per se reduced basal PAI-1 but produced only minor attenuation of basal t-PA production. These results could be explained by the existence of a basal $G_{\alpha i}$-protein inhibitory tonus on adenylate cyclase, which corroborates other studies demonstrating reduced cAMP following PTX addition (Eguchi 1993, Manolopoulos 1995a). When thrombin was added to cells that had been preincubated with PTX, both the induction of t-PA and PAI-1 were decreased. The results indicated that thrombin signals were transducted through a $G_{\alpha i}$ /$\beta\gamma$-pathway. This was in agreement with reports demonstrating thrombin-induced stimulation of the MAP kinase cascade (Wheeler-Jones 1996, Chen 1996) but was at variance with the rapid PGI_2 production being transducted trough the $G_{\alpha q}$ /PKC pathway (Garcia 1991). Studies exploring this pathway in t-PA and PAI-1 production seem to be rare. Pertussis toxin could add only marginal inhibition to forskolin, further decreasing thrombin-induced t-PA and PAI-1 production. This lack of additive effect could indicate that pertussis toxin and forskolin have an inhibitory influence on the same transduction pathway.

Genistein, Vanadate. Transducting signals through a subsequent series of phosphorylating enzymes have been demonstrated to be one of the commonest mechanisms in cellular communication. The MAP or tyrosine kinase cascade could influence several different effectors, such as transcription factors, phospholipases, cytoskeletal and other regulatory proteins. This pathway could also be modulated by "cross-talk" to other signal transduction systems (Cobb 1991, Johnson 1994, Moolenaar 1995, Seger 1995). Genistein, a rather selective tyrosine kinase inhibitor, has been used extensively to block the transduction of signals in the MAP-kinase cascade (Akiyama 1991). The phosphorylating effect of kinases is balanced, in strength and duration, by phosphatases that de-phosphorylate the substrates. Multiple different enzymes, isomers and substrates exist and no simple ying and yan

relation between kinases and phosphatases has been reported (Hunter 1995). Sodium-ortho vanadate is a frequently used inhibitor of tyrosine-phosphatases. However, the intracellular substrates for vanadate are largely unknown, and disparate effects have been obtained in intact cell systems (Gordon 1991, Shimizu 1994).

In the present study, genistein reduced both basal and thrombin-stimulated t-PA and PAI-1 production. These results demonstrated that signals were transduced from the thrombin receptor to the MAP-kinase cascade. The data also proposed the existence of a basal tonus in the MAP cascade. Vanadate had no influence on basal t-PA or PAI-1 production. However, vanadate decreased thrombin-stimulated t-PA and PAI-1 secretion. The results indicated a phosphatase-dependent step in the thrombin transduction pathway.

Genistein and even phosphatase inhibitors have recently been reported to inhibit the transduction of thrombin and other GTP-binding protein signals to the MAP cascade (Clark 1993, v Biesen 1995, Wheeler-Jones 1995, Chen 1996). In another study, cytokines activating tyrosine kinase receptors have been demonstrated to increase PAI-1 and u-PA but to decrease t-PA production from endothelial cells. Genistein reversed the effect of cytokines and reduced basal production. On the other hand, vanadate further increased cytokine induced production but had no effect on basal PAI-1 production (v Hinsbergh 1994). The differences between the present and the study of v Hinsbergh et al might be explained by the use of different agonists. Cytokine activates receptors at the top of the MAP cascade whereas thrombin signals are associated further down, probably at the adaptor protein Shc (Clark 1993, v Biesen 1995). The use of activating serum also diverged between the studies.

This section has discussed the results in papers II-IV, exploring the regulation of t-PA and PAI-1 production from endothelial cells. In the following section, hypothesized signal transduction pathways based on the results in the present study and data from other cell systems will be presented.

Hypothesized signal transduction pathways

–in adult vein endothelial cells

See figure, with figures (1-7) corresponding to the text below.

1) Thrombin, by signalling through its specific cell surface receptor, increased production of t-PA and PAI-1 from endothelial cells (papers II-IV). The thrombin signal diverged already at the GTP-binding protein level, dissociating both $G_{\alpha q}$ and $G_{\alpha i}$ from its $\beta\gamma$-dimers (Brass 1993, 1995, v Obberghen-Schilling 1993).

2) The $G_{\alpha q}$-pathway has been demonstrated to activate PLC and PKC, (Brass 1993, 1995, v Obberghen-Schilling 1993). PMA, which induces long-lasting activation of PKC, increased both t-PA and PAI-1 production (Paper III).

3) Thrombin-induced t-PA and PAI-1 could be reduced by pertussis toxin, indicating thrombin signalling through the $G_{\alpha i}$-pathway (Paper IV).

4) Genistein, an inhibitor of MAP-kinase, reduced thrombin-increased t-PA and PAI-1 production. These results indicated that thrombin transducts signals through the MAP-kinase cascade (Paper IV). The phosphatase inhibitor, sodium ortho-vanadate, had no effect on basal t-PA or PAI-1 production. However, vanadate could reduce thrombin-stimulated t-PA and PAI-1 production (data not published).These

data indicated the presence of a phosphatase-dependent step in the thrombin transduction pathway, corroborating data by Clark (Clark 1993).

5) Forskolin, which increases intracellular cAMP, reduced basal PAI-1 production but had only minimal influence on basal t-PA production. Furthermore, forskolin also decreased PKC-induced PAI-1 production but stimulated PKC-induced t-PA production (Paper III). These data indicated that cAMP had an inhibitory effect on the transduction pathway: a) between PKC and the PAI-1 promotor, and b) between PKC and the receptor or on a parallel pathway regulating t-PA production.

6) Forskolin/cAMP reduced thrombin-induced t-PA production but not PMA/PKC-induced t-PA production. Reduction of the thrombin-effect was not influenced by preincubation with pertussis toxin (paper III-IV). These data indicated that the decreasing effect of forskolin on thrombin-stimulated t-PA production did not act on the $G_{\alpha q}$/PKC pathway, but probably on the $G_{\alpha i}$ pertussis inhibiting pathway. The small GTP-binding protein, Ras, which activates Raf-1 in the MAP kinase cascade, has been reported to be inhibited by cAMP and could be a potential candidate for the observed forskolin-induced t-PA inhibition (Johnson 1994, Post 1996). Forskolin/cAMP also reduced thrombin-induced PAI-1 production. As both basal and PMA-induced PAI-1 production were inhibited by forskolin, it was not possible to construct a hypothesis concerning the $G_{\alpha i}$-pathway inhibition of thrombin-induced PAI-1 production.

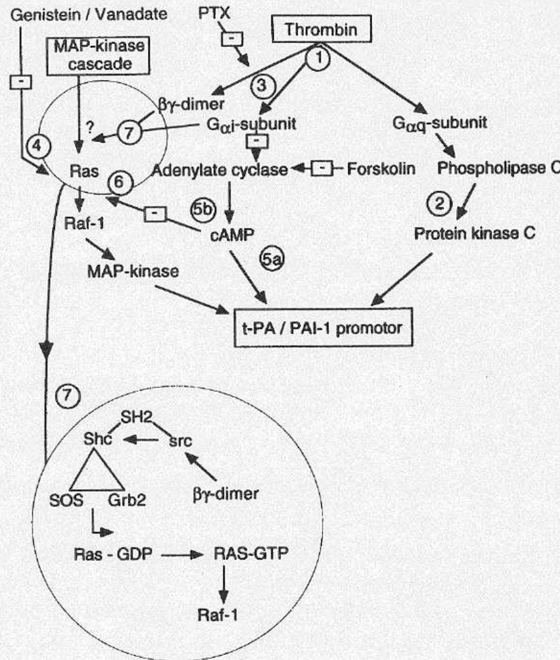


Fig 16. Hypothesized signal transduction pathways, in adult vein endothelial cells. Figures (1-7) corresponding to the text in this section. The pathway from the thrombin βγ-dimer to the MAP kinase cascade is inserted as a magnification.

7) The signal transduction pathway from the thrombin-receptor to the MAP kinase cascade has not been completely described in endothelium. In other cell systems, $\beta\gamma$-dimers dissociated from $G_{\alpha i}$ have been reported to activate Ras in the tyrosine kinase cascade (Corven 1993). Recently, the $\beta\gamma$-dimer has been demonstrated to activate proteins in the Src family. When activated, Src could phosphorylate the adaptor protein Shc. Activated Shc unmasks binding sites for the SH_2 domain containing proteins as Ras and Grb_2. When bound, Grb_2 and the associated SOS protein are brought to the membrane where they could activate Ras. Both PH-homology domains on the $\beta\gamma$-dimer and membrane lipids seem to be involved in the signal transduction (Bong 1995, v Biesen 1995, Bokoch 1996, Post 1996, Chen 1996).

Endothelial heterogeneity

Cultured endothelial cells have been used extensively in in-vitro experiments. When data from different studies have to be compared it is important to realise that the results are dependent on the construction of the experimental model used. One of the most important factors in a cell model is the primary origin of the cells. Human umbilical vein endothelial cells have been frequently used to examine the regulation of t-PA and PAI-1 production. Adult human endothelial vein cells have been used by some authors in an attempt to get closer to the clinical situation (v Hinsbergh 1987, Kooistra 1991, Haegerstrand 1992, Arnman 1993).

Endothelial heterogeneity_

Some characteristics are shared by all endothelial cells. e.g. production of the von Willebrand factor (Jaffe 1973b), prostacyclin, nitric oxide (Moncada 1990), expression of angiotensin-converting enzyme, VEGF-receptor and E-selektin (Risau 1995). Other endothelial qualities demonstrate great inter-organ variation. In the literature there are several reports demonstrating endothelial organ heterogeneity:

- differences between species (Taylor 1987, Bartha 1988, v Hinsbergh 1988).
- differences between individuals of the same species (Levin 1984, v Hinsbergh 1988, Chandler 1991).
- differences between anatomical locations (v Hinsbergh 1987, Taylor 1987, Wojta 1989, Iba1991, Chandler 1992, Schrauwen 1994, Manolopoulos1993, 1995a,b)

Qualitative differences

Intracellular origin-heterogeneity_

Hypothetically, several intracellular levels could be of interest in an attempt to explain these differences. Heterogeneity has been described between:

- cells with different sets of GTP-binding proteins that could be dissociated by the receptor (Birnbauer 1992, Bernstein 1992, Hepler 1992).
- cells with different isoforms of intracellular enzymes such as adenyl cyclases (Iyengar 1993, Manolopoulos 1995), kinases (Johnson 1994, Nishizuka 1995) and lipases (Lee 1995, Roberts 1996). These different isoforms have both diverging regulatory and substrate-affinity patterns.
- cells with a different construction in their DNA-promotor regions. The rat t-PA promotor contains a CREB /cAMP responding element, whereas the human and mouse promotor in the corresponding position has a modified CRE-like sequence responding to AP-1/ PKC. Consequently, the rat, but not the human or mouse cells, increase their t-PA production when stimulated with cAMP (Medcalf 1990, Ohlsson 1992).

External dynamic heterogeneity
The characteristics to be expressed at a certain time by a specific cell are determined by several mechanisms. The state of cellular phenotype, being in dynamic interaction with the surrounding tissue, regulates the uncovering of genes (Pepper 1993, Risau 1995, Edmondson 1996).
External signals could regulate:

- receptor sensitivity (Hausdorff 1990, Brass 1995, Runge 1995),
- desensitization of intracellular transduction enzymes (Duniec 1990, Nishizuka 1995)
- interactions between signal transduction pathways ("cross-talk") (Newman 1989, Garcia 1992, Johnson 1994, Bokoch 1996),
- prolonged stimulation could also affect the cellular synthesis of different GTP binding proteins and enzymes (Duniec 1990, Campell 1993, Eschenhaugen 1992).

Consequently, the incoming receptor signals could be interpreted in a variety of ways and evoke different responses depending on the type of cell or tissue. On the other hand, these differences might provide a tool for tissue-specific pharmacological modulations

In the present study (paper III), qualitative differences in stimulated production of t-PA and PAI-1 were demonstrated between umbilical and adult endothelial cells. Forskolin-modulating PMA, LPS or thrombin-stimulated t-PA expression differently in umbilical and adult cells respectively. Thrombin signal transduction through $G_{\alpha i}$-protein and tyrosine kinase pathways has been explored both in adult cells (paper IV) and umbilical cells (not published), however, no differences could be detected.

Quantitative differences

The production of t-PA and PAI-1 from endothelial cells of different origin is a function of qualitative differences including cell origin, phenotype, preceding history and is also dependent on the state of ongoing stimulation.

In this study (paper III), we presented quantitative differences, comparing the basal production of t-PA and PAI-1. Umbilical cells produced higher quantities of both t-PA and PAI-1 than adult cells. Differences have been demonstrated earlier. However, Arnman (Arnman 1993) presented higher t-PA production from adult, compared with umbilical cells. Furthermore, in paper IV we found PAI-1 levels in adult cells comparable with levels in umbilical cells in paper III. This discrepancy probably reflects the great quantitative variations between human endothelial cells of different origin in vitro (v Hinsbergh 1988) or between different individuals in vivo (Greén 1986, Chandler 1991).

Thus, to present reliable results of quantitative differences between cells from different origins, data have to be collected from a large number of cultures representing different individual veins.

Summary

There is still no consensus about the type of cell to be used in vascular in-vitro experiments. As endothelial cells from different origins in the body have proved to be quite heterogeneous it cannot be presumed that cells from only one of these origins could be representative of all types of vessels. Even if a great deal of data could be generalized, the cell type being the most relevant for an in-vitro experiment, depends on the vascular segment of clinical interest. Furthermore, it is very important to keep the experimental conditions identical throughout an experimental series in order to be able to compare the results.

SUMMARY

The main experimental findings were:

1. The production of prostacyclin from endothelial cells was increased by the adrenergic agonist isoprenalin.

2. The β-adrenoceptor blockers, propranolol or metoprolol, synergistically increased endothelial prostacyclin production when co-incubated with isoprenalin. Propranolol or metoprolol per se had no effect.

3. The production of tissue plasminogen activator (t-PA) from umbilical and adult endothelial cells was increased by phorbolester and thrombin. In high concentrations, endotoxin increased the production from umbilical but not from adult cells. Forskolin, isoprenalin, ephedrine, Na-nitroprusside or endothelin-1 had no influence on the production of t-PA.

4. The production of plasminogen activator inhibitor type-1 (PAI-1) from umbilical and adult endothelial cells was increased by phorbolester and thrombin. In high concentrations, endotoxin increased the production from umbilical but not from adult cells. Forskolin decreased PAI-1 production. Isoprenalin, ephedrine or endothelin-1 had no influence on the production of PAI-1. Na-nitroprusside had a weak increasing effect.

5. The protein kinase C inhibitor H7 decreased t-PA and PAI-1 production which had been induced by endotoxin, thrombin or phorbolester in both umbilical and adult cells.

6. Forskolin decreased PAI-1 production which had been induced by endotoxin, thrombin and phorbolester both in umbilical and adult cells.
   On the other hand, the effect on t-PA production diverged between cells of different origin when endotoxin, thrombin or phorbolester were co-incubated with forskolin.

7. Thrombin-induced tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1) production from adult endothelial cells were decreased by pertussis toxin or genistein, representing $G_{\alpha i}$-protein and tyrosine kinase pathway inhibitors respectively.

CONCLUSIONS

1. The production of prostacyclin from endothelial cells is under adrenergic control, modulating the effect of β-adrenoceptor blockers.

2. Signal transduction through adenylate cyclase, protein kinase C, $G_{\alpha i}$-protein and tyrosine kinase pathways is critical in the regulation of t-PA and PAI-1 production from endothelial cells.

3. Interference with adenylate cyclase could be one way of modulating the balance between coagulation and fibrinolysis in the vessel.

4. Endothelial cells of different origin regulate the production of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type-1 (PAI-1) differently.

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