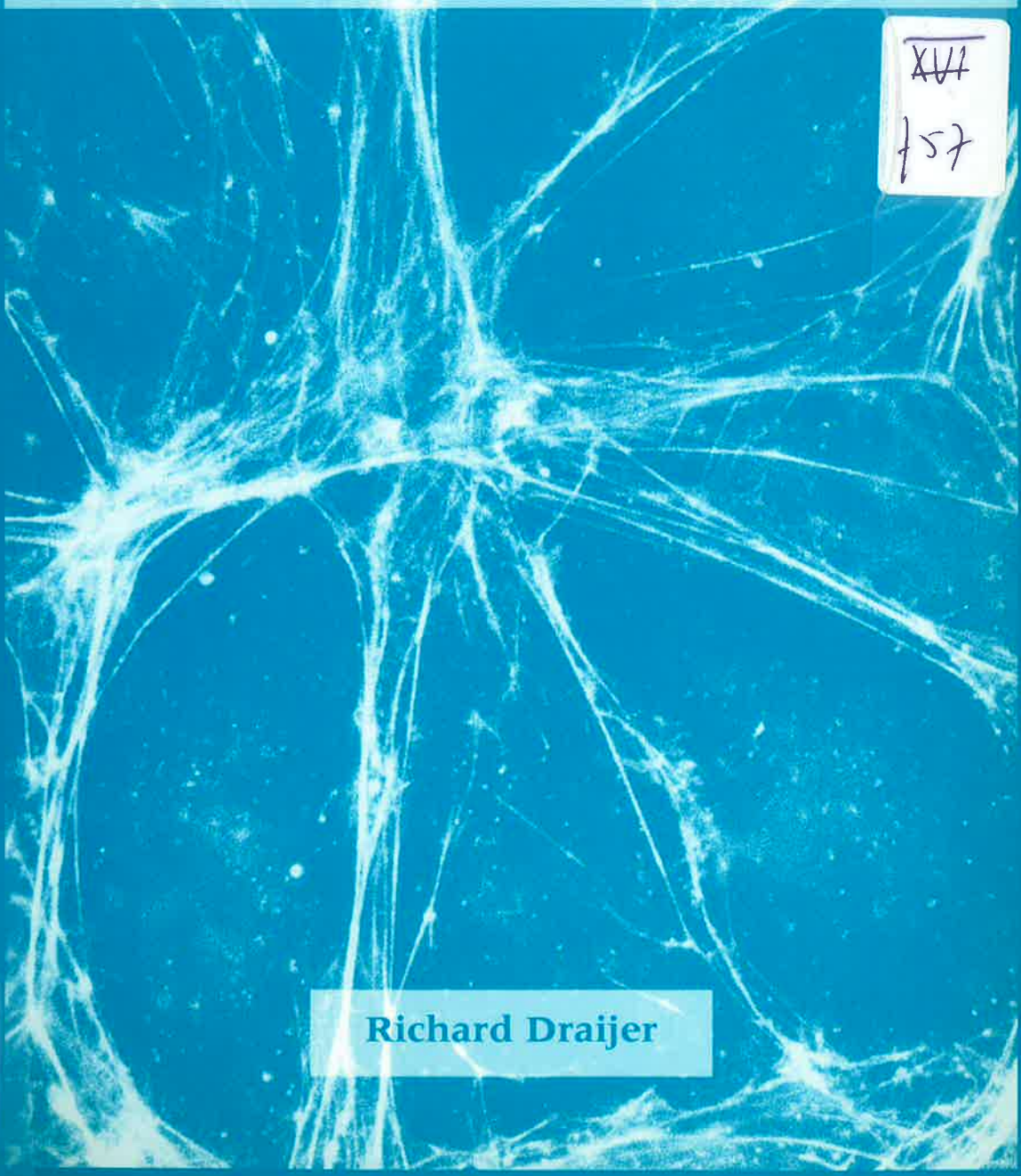
Regulation of human endothelial barrier function by cyclic nucleotides, nitric oxide and protein kinases.



Richard Draijer

Regulation of Human Endothelial Barrier Function
by
Cyclic Nucleotides, Nitric Oxide and Protein Kinases

Stellingen

behorende bij het proefschrift

Regulation of endothelial barrier function
by cyclic nucleotides, nitric oxide and protein kinases.

1. Het gunstige effect van stikstof oxide op de endotheel barrière dient, naast de vasodilaterende werking van dit gas, onderkend te worden, indien men wil verklaren hoe inhalatie van stikstof oxide vermindering van long oedeem kan bewerkstelligen (Rossaint et al, N Engl J Med, 1993, 328, 399-405; dit proefschrift).

2. Het feit dat calcium-ion afhankelijke endotheel cel contractie beïnvloed wordt door cGMP, heeft gevolgen voor andere endotheliale processen die door calcium-ionen gereguleerd worden (dit proefschrift).

3. Verbetering van de endotheel barrière door ß-adrenerge stimulatie is slechts een kwestie van tijd (dit proefschrift).

4. De aspecificiteit van proteïne kinase C remmers heeft geen remmende invloed op het gebruik ervan.

5. Het belang van stikstof oxide bij de erectie (Rajfer et al, N Engl J Med, 1992, 326, 90-94), kan betekenen dat het microvasculaire endotheel van de voorhuid niet als representatief beschouwd mag worden in het onderzoek naar de effecten van stikstof oxide op endotheel cellen.

6. De term "knock-out" bij knock-out muizen is veelal meer van toepassing op de onderzoeker en zijn werkhypothese dan op de muizen zelf.

7. Specifieke remmers zijn net als fotomodellen: hoe jonger, hoe aantrekkelijker.

8. De stelling dat "de mensheid de huidziekte van de aarde vormt" (een uitspraak van J.E. Lovelock in verband met zijn "Gaia-hypothese"), heeft gezien de enorme hoeveelheid ruimtevaart afval die we buiten de dampkring achterlaten, een bredere implicatie.

9. Het broeikas effect ten gevolge van de uitstoot van methaan gas door tropische kakkerlakken (Hackstein et al, PNAS, 1994, 91, 5441-5445) kan vertraagd worden door het tropisch regenwoud te kappen.

10. Evolutie heeft het Gods-begrip gecreëerd.

11. De aanschaf van wasmiddelen met "micro waskracht" valt te ontraden, daar de waskracht één miljoenste is van die van voorheen.

12. "All revved up with no place to go" van de popzanger Meat Loaf kan als het lijflied van de gepromoveerde AIO gezien worden.

Leiden, 9 januari 1996 Richard Draijer

Regulation of Human Endothelial Barrier Function by Cyclic Nucleotides, Nitric Oxide and Protein Kinases

Proefschrift
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RICHARD DRAIJER
geboren te Zandvoort in 1964

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Cover: Actin filaments in endothelial cells under basal- and thrombin-stimulated condition.

Aan mijn moeder
Voor Marieke

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. Endothelial functions and properties

The endothelium covers the luminal surface of blood vessels, thereby separating blood constituents from interstitial tissue. Originally viewed as a passive barrier, the endothelium is now regarded as an active regulator of vascular homeostasis (Figure 1).[20,53,63,89]

- The endothelial lining is under normal circumstances nonthrombogenic: the blood coagulation cascade and the adherence of platelets to the vessel wall are controlled by anticoagulant molecules derived from or located on the endothelium. The plasma protein antithrombin III binds to glycoaminoglycans on the endothelial cell (EC) surface, which accelerates the inactivation and cleareance of the clotting enzyme thrombin. Additionally, the endothelial receptor protein thrombomodulin catalyzes the activation of protein C by thrombin. The activated protein C inactivates the cofactors factor Va and VIIIa, by which the coagulation cascade is interrupted. Furthermore, the endothelium is able to synthesize tissue factor inhibitor and protein S, a cofactor for activated protein C. Platelet adhesion and activation is prevented by heparan sulphates on the endothelium and by the endothelial synthesis of nitric oxide and prostacyclin. Finally, when fibrin is generated in the vascular lumen the endothelium can release tissue-type plasminogen activator (t-PA), thereby activating the extrinsic fibrinolytic pathway. On the other hand, coagulation is promoted by von Willebrand factor (vWF) which is constitutively secreted by ECs and forms a carrier for coagulation factor VIII and an anchor for platelets at the subendothelium. ECs also secrete plasminogen activator inhibitor-1 (PAI-1) and synthesize platelet activating factor (PAF), which is a potent mediator of platelet aggregation.
- The vascular tone is regulated by vasodilators (nitric oxide and prostacyclin) and vasoconstrictors (endothelin) secreted by ECs. Formation of vasoconstrictors can be promoted (for instance, cleavage of angiotensin I to the vasoconstrictor angiotensin II by the angiotensin-converting enzyme located at the surface of ECs), or inhibited by ECs (such as bradykinin which is metabolized by endothelial kininase II). Vasoactive peptides (substance P), amines (norepinephrine, serotonin) and adenine nucleotides (ADP, ATP) are also degraded by ECs.

- Neovascularization is under the control of ECs and is involved, for instance in wound healing and tumour growth. Endothelial cells respond to angiogenic factors, such as vascular endothelial cell growth factor (VEGF) and fibroblast growth factors (acidic- and basic FGF), which trigger the enzymatic degradation of the basement membrane underneath the endothelial monolayer, successively followed by the migration of ECs in the interstitial tissue, and proliferation and maturation of ECs into newly formed capillaries. The supporting basement membrane consists among other proteins of collagens, proteoglycans and heparan sulphates and is produced by the EC.
- During wound healing and inflammation the endothelium directs blood cells to the appropriate sites (see next section).
- One of the primary functions of the endothelium is the formation of a physical barrier between blood constituents and the extravascular tissue. Endothelial permeability is increased by numerous vasoactive agents, such as bradykinin, thrombin, histamine, PAF and adenosine triphosphate (ATP), whereas it is decreased by others, such as serotonin and ß-adrenergic stimuli. The next section will discuss the properties of the endothelial barrier function under normal and stimulated conditions.

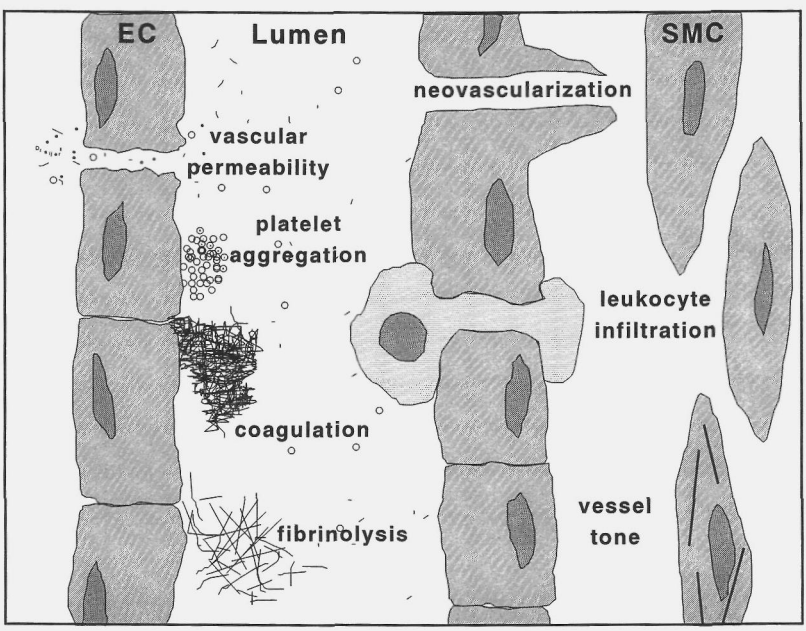

Figure 1: Schematic representation of a blood vessel depicting endothelial properties and functions. EC, endothelial cell; SMC, smooth muscle cell.

1.2. Endothelial permeability under pathophysiological conditions

According to the Starling hypothesis fluid transfer is determined by the balance of the hydrostatic and osmotic forces across the endothelium. Under normal conditions, the transendothelial hydrostatic pressure gradient promotes fluid filtration, while the transendothelial colloid osmotic pressure gradient opposes fluid filtration. Intercellular gap formation disturbs the equilibrium of the Starling forces, because large amounts of plasma proteins flow into the interstitial tissues, leading to loss of the colloid osmotic pressure gradient.[24] The exchange route and quantity of fluid and macromolecules can change dramatically when endothelial cells are activated or injured under particular circumstances or diseases:

(1) Vascular leakage of postcapillary venules in inflammation.

- Vascular leakage of postcapillary venules occurs following exposure to inflammatory mediators, such as histamine, bradykinin, leukotrienes, PAF, immune complexes, complement fragments, TNFα, interleukins and free radicals. These mediators normally cause transient leakage, but sequential release of several mediators by neutrophils, monocytes or lymphocytes sustains the effect. Neutrophil accumulation at sites of inflammation probably causes vascular injury and local edema.[26,65] Endothelial cells protect themselves by releasing adenosine, which inhibits neutrophil adherence and oxidant production, and by endothelial anti-oxidant enzymes, which scavenge toxic oxygen radicals generated by neutrophils. Blood monocytes and tissue macrophages release products, such as TNFα and IL-1, that affect EC function. TNFα causes edema indirectly by activation of neutrophils. Eventually the acute inflammation can convert into immune inflammation. The cytokines TNFα and IL-1 induce the expression of adhesion molecules for leukocytes, such as E-selectin and ICAM-1, and the extravasation of leukocytes is facilitated by morphological changes of the EC. Expression of major histocompatibility complex (MHC) class II on ECs is induced when the ECs are activated by the lymphocyte-derived interferon-γ. MHC II binds to foreign antigens, which is recognized by antigen-receptors on T helper cells.
- Inflammatory reactions are of great concern in patients with adult respiratory distress syndrome (ARDS). In this disease pulmonary vascular leakage is one of the severe consequences.[5,9,68]
- Glomerular injury can result from inflammatory responses by the EC.[83] Generalized edema and leakage of large proteins is a common feature in Nephrotic Syndrome, associated with several renal diseases.
- Edema of the airway mucosa in asthma is attended by bronchospasm, but infiltration of eosinophils and lymphocytes also indicates an inflammatory cause.

(2) Vascular leakage associated with elevated blood pressure.

- Cardiac heart failure can cause acute pulmonary edema, induced by a sudden rise in the left ventricle pressure that results in a rapid movement of plasma fluid through pulmonary capillaries into the interstitial tissue and alveoli.
- A relatively hypoxic athmosphere at high altitude may cause High-Altitude Pulmonary or Cerebral Edema, which is attended by increased vascular resistance and pressure.

(3) Vascular leakage associated with tumours.

- The vasculature in the vicinity of tumours can leak by factors that are released by the tumour cells, such as vascular permeability factor (VPF, which is identical to VEGF). For instance, increased permeability of peritoneal microvessels correlates well with the VPF concentration in peritoneal fluid.[57] A leaky endothelium facilitates the infiltration of circulating cancer cells, which results in metastasis. Furthermore, the release of angiogenic factors can induce the formation of new blood vessels around the tumour, which supply nutrients to the cancer cells.

(4) Vascular leakage associated with EC damage.

Leakage of arterioles, capillaries and venules can be caused by EC damage resulting from severe trauma, thermal injury or toxicity.

- Oxygen toxicity can occur during hyperoxia, probably by overproduction of oxygen free radicals, and can damage the pulmonary vasculature, thus increasing endothelial permeability.[96]
- Dying and necrotic tissue may become a source of inflammatory mediators. In a rat model it has been demonstrated that aseptic necrosis induced immediate and transient leakage of arterioles, capillaries and venules, followed by leakage restricted to the venules, and finally a delayed capillary leakage.[33]
- Interstitial edema occurs in hyperacute rejection of vascularized grafts, initiated by binding of xenoreactive antibodies to donor ECs and by the activation of complement. It has been demonstrated in an *in vitro* model that the antibodies and complement alter the EC shape and disrupt the monolayer integrity. The subsequent gap formation requires components of the complement system and is not caused by cytotoxicity.[71]
- Protein deficiency caused by starvation or malnutrition can result in generalized edema.
- Several factors may be involved in vascular leakage in Diabetes Mellitus: the persistent hyperglycaemia causes auto-oxidation of glucose, and glycosylation of proteins may generate free radicals. The synthesis of heparan sulphates by ECs, which is important as a negatively charged barrier on ECs, is impaired.[95]
- The endothelial function is impaired in atherosclerotic vessels. Release of nitric oxide is diminished and signal transduction via G proteins is inhibited.[16,59] It is suggested that oxidized LDL interferes with the availability of L-arginine, which forms the substrate for NO

production. Chronically elevated levels of LDL, as in patients with Hypercholesterolaemia, may affect the endothelial barrier. The increased permeability in atherosclerotic vessels[81] is partly related to focal leakage spots.[82] These focal leakage spots represent predominantly EC mitosis[10] and occasionally defective junctional complexes between aorta ECs.[29] A decrease of heparan sulphate proteoglycans of the basal membrane and increased macromolecular permeability are observed with high LDL concentations *in vitro*.[25] Branched regions of arteries are more permeable for macromolecules than unbranched regions.[34] This difference is not caused by different mitotic frequency or by increased vesicular transport, because the amount of vesicles is lower in branched regions, but is probably due to the frequently observed open junctions in the branched regions. Low density lipoprotein (LDL) preferentially accumulates in the branched regions, which may initiate lesions in the vessels.[28]

The foregoing indicates that increased vascular permeability is caused, when restricting to the endothelium, by an impaired endothelial function, endothelial injury or intercellular gap formation. The intracellular mechanisms that are involved in endothelial gap formation were studied in this thesis.

1.3. Vascular barrier properties of the endothelium

Four types of endothelium are recognized. A continuous endothelium lines the majority of blood vessels, including arteries, arterioles, veins, venules and capillaries of skeletal muscle, myocardium, skin and connective tissue. A very tight type of continuous endothelium is found in capillaries of the brain and spinal cord, where it forms the blood-brain and blood-spinal barrier. The endothelium of capillaries of most internal organs and visceral tissues are fenestrated, characterized by fenestrae (openings) sometimes covered by a diaphragm. In glomeruli the basal membrane and its (negative) surface charge form in fenestrae the main barrier. Open or discontinuous endothelium lines the sinusoids of the liver, spleen and bone marrow. Relatively large particles can pass freely over this nonselective endothelial barrier, which only obstructs the passage of particles as large as chylomicrons.

Several molecular exchange and transport pathways through the endothelial monolayer are distinguished (Figure 2).[69,90] Exchange of molecules that do not easily penetrate the cell membranes occurs mainly via the paracellular route: gaps between cells, chains of vesicles and open fenestrae. The endothelial cell barrier is strengthened by the basal lamina underneath the cells, that forms a layer of fine collagenous fibres interconnected by laminin, negatively charged heparan sulphate proteoglycans (HSPG) and other glycoproteins. This protein meshwork provides tissue support and molecular sieving. Furthermore, the ECs are covered with albumin-bound glycoproteins and glycosaminoglycans forming the glycocalyx that extends into the cell junctions.[69,100] Experiments in which the charge of the glycocalyx

was neutralized or the exchange of charged and noncharged molecules was evaluated, revealed that the movement of negatively charged molecules is restricted by the glycocalyx.[85]

In conclusion, the permeability of macromolecules decreases as the lipid-insolubility of the molecules increases, the molecules become more negatively charged, and the molecular size increases.

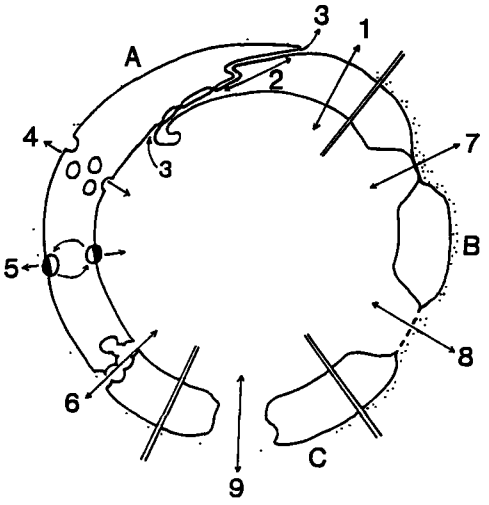

Figure 2: Molecular exchange and transport pathways through monolayers of continuous and discontinuous endothelium. (1) Free diffusion of small gaseous molecules (oxygen), (2) lateral exchange of small lipid molecules along the cellular lipid bilayers, (3) exchange of molecules through the narrow clefts of neighbouring EC, (4) vesicular exchange: the endothelial surface is covered with numerous vesicles (outer diameter of 60-70 nm), mainly in opened condition at the luminal site, (5) receptor-mediated vesicular transport, mainly operational in the very tight continuous endothelium of the brain, (6) vesicular fusion may lead to trans endothelial channels through which solutes and macromolecules travel, (7) exchange through fenestrae with closed and (8) open diaphragm, (9) exchange of large macromolecules through gaps in sinusoidal endothelium and through intercellular gaps in postcapillary venules during inflammatory response. A: exchange pathways of continuous endothelium; B: Fenestrated endothelium; C: Sinusoidal endothelium and activated postcapillary venules (from: Van Hinsbergh[90]).

The strength of the barrier, particularly of the continuous type of endothelial cells, depends on the cell-cell and cell-matrix interactions. Integrines are heterodimeric glycoproteins on the surface of the EC, which associate with collagen, laminin or fibronectin, thus anchoring the EC to the extracellular matrix. Furthermore, focal attachment sites play an important role in cell-matrix adhesion. These contact sites are specialized membrane areas where the cell binds to the matrix and involves several proteoglycans. Cell-cell adhesion is promoted by cadherins, which form a calcium-dependent homophilic binding, platelet-endothelial cell adhesion molecule-1 (PECAM-1), which binds calcium-independently, and probably integrins (ß1).[1] The transmembrane VE-cadherin is connected via intracellular

catenins and plakoglobulin to vinculin, which is linked to filamentous actin.[14,48] Other cell surface receptors or secreted proteins may be involved in the endothelial cell shape, such as lectin-like cell adhesion molecules (selectins)[35] and SPARC.[21]

Regional narrowings (interendothelial junctions) are important for cell-cell interactions. Gap junctions are regions of close membrane oppositions in which channels of opposed membranes are linked. The hydrophylic channels are built up by hexameric proteins (connexins) and allow small molecules (~1 kDa) to pass to adjacent cells. They provide a low-resistance pathway for intercellular metabolic communication between ECs and probably between ECs and other vascular cells.[4,13,44] The closely opposed cell membranes in tight junctions determine the apical-basal polarity of ECs and endothelial barrier function. ZO-1, ZO-2, occludin, cingulin and small GTPase are proteins found in these regions, and are thought to be involved in the junctional integrity.[11,32,101]

1.4. Cellular mechanisms involved in intercellular gap formation

Massive leakage of macromolecules during the acute inflammatory response, in particular in the postcapillary venules, occurs by contraction of the marginal areas of ECs, leading to intercellular gap formation.[50,52,84] The contraction involves the interaction of non-muscle myosin and F-actin, particularly located at the periphery of the EC.[75,78] F-actin filaments anchored at the basolateral site and spanning the EC are merely involved in sticking ECs to the subendothelial surface and help to resist the fluid shear stress.[23] Contraction of ECs requires cytoplasmic calcium ions ($[Ca^{2+}]_i$), the calcium-binding protein calmodulin, adenosine triphosphate (ATP) and myosin light chain (MLC) kinase.[97,98] In analogy with smooth muscle cells the MLC kinase may be activated by a Ca^{2+}-calmodulin complex. The activated MLC kinase can phosphorylate, by hydrolyzation of ATP to ADP, the 20 kDa MLC, which is located at the myosin head.[2,31] Phosphorylation of MLC promotes binding of myosin to actin filaments and initiates the actomyosin complex ATPase activity.[87] The liberated chemical energy after ATP hydrolysis is the driving force for the conformational change of the crossbridge between myosin and actin filaments, leading to filament movement.

1.5. Evaluation of the endothelial barrier function in an *in vitro* model

Endothelial cells isolated from their natural environment and cultured in an *in vitro* system have particular advantages for studying mechanisms involved in the regulation of endothelial permeability. Several agents induce *in vivo* vasorelaxation (NO, prostacyclin) or vasoconstriction (PAF, histamine) in the investigated vascular bed or adjacent vessels. Permeability of capillaries may increase by relaxation of arterioles, which leads to an increased capillary hydrostatic pressure. Furthermore agents may be cleared or metabolized by blood constituents or ECs before reaching the target site. Local high concentrations of

mediators, such as massive release of ADP by coagulated platelets,[22] are more easily to mimick in an *in vitro* model than in a whole animal. The *in vitro* data should of course be compared to *in vivo* data to obtain maximal information.

Because vascular leakage mainly occurs in postcapillary venules, culture of this type of EC would be most desirable for studying endothelial permeability. Unfortunately, isolation and culture of microvascular EC is difficult and time-consuming. In contrast, human EC from the umbilical vein and artery are easy to obtain without contamination by a mild digestion of the vessels with collagenase. These EC cultured on porous filters have extensively been used and characterized for permeability studies in our laboratory.[36,37] They fulfil several *in vivo* conditions when cultured to tight monolayers on fibronectin-coated polycarbonate Transwell^R filters:

(1) The ECs are extensively characterized by EC markers, such as vWF in granules, uptake of acetylated-LDL, membrane markers CD31 (PECAM-1) and VE-cadherin and free of contaminating smooth muscle cells (smooth muscle actin) or fibroblasts. The cultured ECs secrete t-PA, u-PA and prostacyclin and respond to inflammatory mediators, such as TNFα, IL-1, histamine and thrombin.

(2) The transendothelial electrical resistance (TEER, about 15 to 20 $\Omega.cm^2$) resembles *in vivo* measurements in arteries. The TEER is thought to represent a measure for the endothelial barrier for ion fluxes restricted by tight junctions.

(3) The integrity of the monolayers is dependent on the presence of albumin and extracellular Ca^{2+} ions. Both elements are necessary for building up the endothelial barrier, because albumin forms part of the glycocalyx on the EC and the Ca^{2+} ions are cofactors for calcium-dependent intercellular connective proteins (cadherins).

(4) The cultured monolayers display clear molecular sieving characteristics. Small molecules like galactose and sucrose easily travel across the monolayers, whereas diffusion of mean sized molecules like dextrans of 4.4 to 40 kDa or horseradish peroxidase and larger molecules like LDL is highly restricted. The monolayers are more repulsive to negatively charged than to uncharged molecules.

(5) Electron microscopy revealed the presence of tight and gap junctions between adjacent cells. Tight junctions are thought to be involved in cellular adhesion and to maintain the endothelial barrier. Intercellular exchange of small molecules takes place in the gap junctions.

In addition to human umbilical artery and vein ECs, human ECs derived from large vessels, including aorta, iliac vein and pulmonary artery, and recently from the foreskin microvasculature have been cultured with similar barrier characteristics (see chapter 2 of this thesis). Thus, these endothelial monolayers give the opportunity to evaluate which intracellular signal pathways determine the endothelial barrier function, and whether differences exist in the regulation of permeability between different types of ECs.

Thrombin was used to mimick vascular leakage. This clotting enzyme proteolytically cleaves its receptor, creating a new amino terminus that acts as a tethered ligand.[92] The modified receptor then activates several pathways, including the activation of a G protein-linked phospholipase C, which causes formation of IP_3 and diacylglycerol and elevation of the cytoplasmic Ca^{2+} ion concentration (see next section). In addition to its effect on blood coagulation at sites of endothelial damage, thrombin triggers the EC to express adhesion molecules (P-selectin), to synthesize and release vasoactive mediators (prostacyclin, NO, PAF, t-PA) and growth factors (b-FGF).[67] Furthermore, several *in vitro* studies demonstrated that thrombin induces an increase in endothelial permeability, which is attended by intercellular gap formation.[3,19,39,47,72] We used this last characteristic to evaluate the endothelial barrier function under stimulated conditions. Horseradish peroxidase, which is demonstrated to pass the endothelial monolayer *in vivo* via the paracellular route,[29] and dextrans of various molecular masses labelled with fluorescein-isothiocyanate were used as tracer molecules.

1.6. Potential cellular sites for regulation of endothelial permeability

Because Ca^{2+} ions are involved in endothelial contraction, modulation of the $[Ca^{2+}]_i$ may have potentially therapeutic effects on vascular leakage. The $[Ca^{2+}]_i$ in ECs is increased by agents that induce gap formation and vascular leakage, such as ATP, histamine, PAF, bradykinin and thrombin. These vasoactive substances bind to specific cell membrane receptors coupled to G proteins, which activates phospolipase C (PLC). PLC can hydrolyse phosphatidylinositol-4,5-bisphospate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 induces the release of Ca^{2+} ions from intracellular stores and promotes extracellular Ca^{2+} ion influx (Figure 3). The involvement of the protein kinase C activator (PKC) diacylglycerol in endothelial contraction has not been established yet, but several studies associate PKC activation with increased endothelial permeability. The $[Ca^{2+}]_i$ can increase, besides via the above mentioned IP_3 pathway, by calcium-influx via passive leakage through low-conductance cation channels and electrogenic Na^+/Ca^{2+} exchange (recently observed in cardiovascular ECs).[41] The extent of calcium influx depends on the state of the intracellular Ca^{2+} stores, i.e. empty Ca^{2+} stores trigger calcium entry. Ca-ATPase pumps associated with the cell membrane and with intracellular Ca^{2+} stores remove Ca^{2+} ions from the cytoplasm. The contribution of other mechanisms in ECs such as voltage-gated channels, stretch activated channels, channels directly coupled to receptors or secondary messengers (IP_3) has been debated.[12,74,88] Regulation of the $[Ca^{2+}]_i$ in ECs by interference in one of these pathways may be one major tool for controlling endothelial permeability.

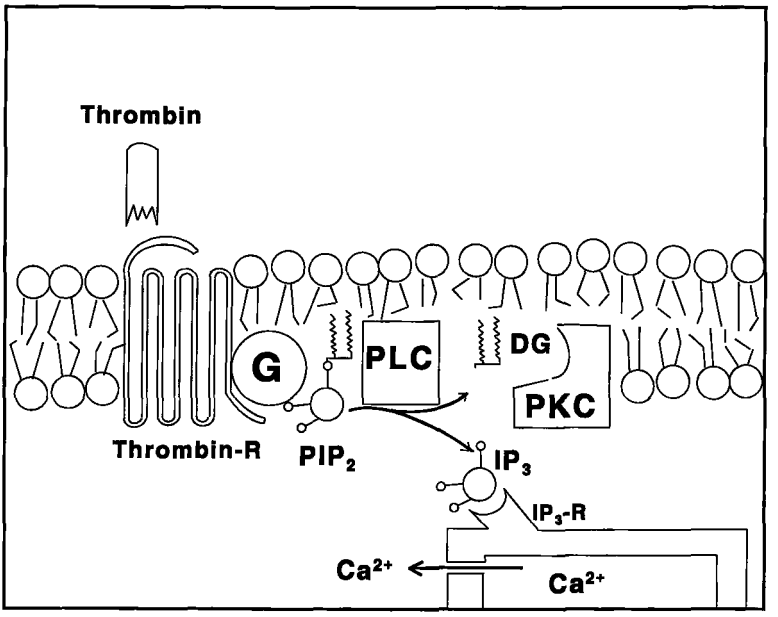

Figure 3: Elevation of the intracellular calcium ion and diacylglycerol concentration by thrombin-stimulation. The proteinase thrombin cleaves its receptor creating a new amino terminus that acts as a tethered ligand and activates its own receptor. Phospholipase C (PLC) is than via Gq-proteins activated and hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). DG is the endogenous protein kinase C (PKC) activator. IP_3 receptors are linked to intracellular Ca^{2+} stores that release Ca^{2+} ions upon IP_3-binding.

Agents that reduce endothelial permeability *in vitro* often concomitantly increase cytoplasmic adenosine 3',5'-cyclic monophosphate (cAMP). Prostacyclin, a prostaglandin synthesized by ECs from the diacylglycerol-derived fatty acid arachidonic acid, and the stable analogue iloprost increase cAMP in ECs and reduce permeability *in vitro*.[38,73] ß-adrenergic receptor stimulation by catecholamines (epinephrine, norepinephrine) or specific ß-adrenergic stimuli (isoproterenol, salbutamol, formoterol) also induce cAMP formation (Figure 4) and improve the endothelial barrier function (see chapter 2).[38,73,102] The ß2-subtype particularly is involved. The ß-adrenergic receptors are linked to adenylate cyclase by G-proteins.[40] Both direct activation of the Gs-protein by cholera toxin or the adenylate cyclase activator forskolin increase the intracellular cAMP concentration. Cell membrane-permeable cAMP analogues, such as 8-bromo-cAMP and dibutyryl-cAMP, mimick an intracellular cAMP increase and also reduce endothelial permeability.[38,80] Additionally, adenosine decreases the endothelial permeability by activation of the A_2-purinoceptor on ECs, which is linked to adenylate cyclase.[27] Adenosine can be released by ECs, eventually as a product from extracellular ATP, ADP or AMP. These adenosine nucleotides are namely metabolized by

ectonucleotidases on ECs (by nucleotide triphosphatase, -diphosphatase and 5'-nucleotidase, respectively), to adenosine.[22,64] cAMP formation may also be involved in feedback-mechanism after contractile responses, as during stimulation with histamine. EC contraction is induced via the histamine H1-receptor, but is probably counterregulated by cAMP formation via H2 stimulation.[86] Attenuation of rapid cAMP-degradation by inhibition of phosphodiesterases (PDE)-activity, offers another opportunity to increase intracellular cAMP. Several classes of PDE exist which are differently regulated and degrade cAMP and guanosine 3',5'-cyclic monophosphate (cGMP) with various affinities.[58] The cAMP-degrading PDE are regulated by Ca^{2+}/calmodulin (subtype I), stimulated by cGMP (II), inhibited by cGMP (III) or regulation is unknown (IV). cAMP can activate cAMP-dependent protein kinase (cAMP-PK), a protein which probably plays a crucial role in the cAMP-mediated reduction of endothelial permeability. Addition of the cAMP-PK agonist adenosine cyclic 3'5'-phosphorothioate (Sp-cAMPS) to endothelial cell monolayers reduces permeability.[80] cAMP-PK phosphorylates a broad spectrum of protein substrates[93] of which one is the MLC kinase.[54] MLC kinase activity is reduced by this cAMP-mediated phosphorylation, thus reducing MLC phosphorylation and EC contraction (see previous section).

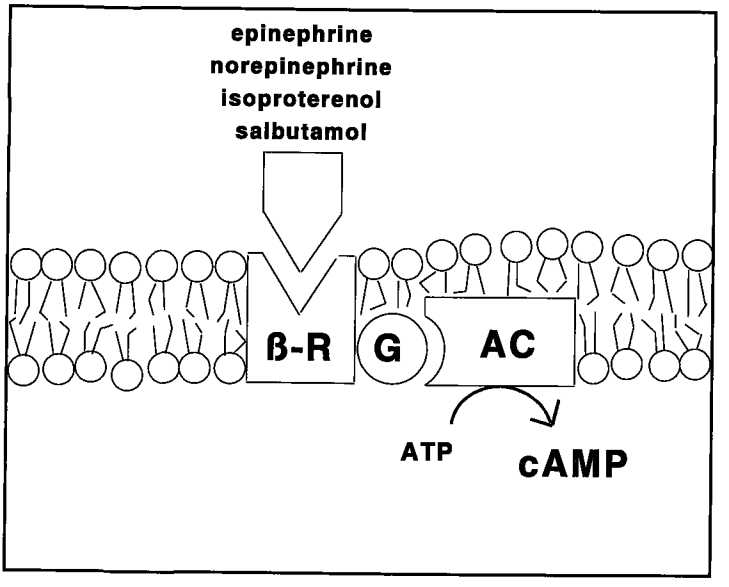

Figure 4: Formation of cAMP by ß-adrenergic stimulation. The ß-adrenergic receptor (ß-R) can be stimulated by the catecholamines epinephrine and norepinephrine or the more specific agonists isoproterenol and salbutamol. Binding of the agonists to the receptor causes activation of GTP-binding heterotrimeric G proteins. The $G\alpha_s$-subunit is involved in activation of adenylate cyclase (AC), the enzyme that hydrolyzes ATP to cAMP.

In analogy with smooth muscle cells another second messenger, cGMP, can mediate relaxation of ECs, which probably leads to a reduction in endothelial permeability *in vitro* and in certain organs.[17,55,66] cGMP levels can be elevated in ECs by atrial natriuretic peptide (ANP), which is produced in the cardiac atria and stored in granules in the myocytes.[15] The C-type ANP receptor is probably important for the clearance of ANP from the blood stream, without linkage of biological activity. The 130 kDa B-type receptor contains, however, a transmembrane-spanning region coupled to a particulate guanylate cyclase, which generates cGMP by hydrolysis of guanosine triphosphate.[76,77] Additionally, the intracellular cGMP concentration is affected by agents that increase $[Ca^{2+}]_i$, since the constitutive nitric oxide synthase (cNOS), located in ECs, is Ca^{2+}-dependent.[61] Activated NOS generates NO from L-arginine and NO activates a soluble guanylate cyclase.[30] The soluble guanylate cyclase is also activated by NO donors, such as nitroglycerin, or free radicals that like NO activate the guanylate cyclase by binding to its heme group (Figure 5).

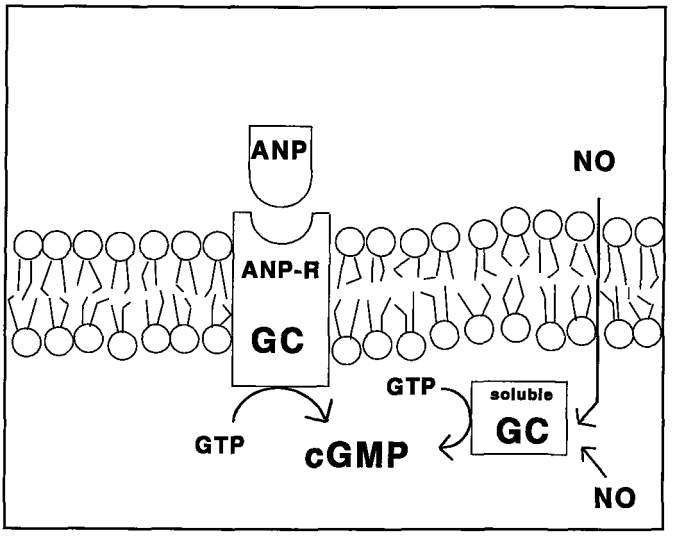

Figure 5: Formation of cGMP by ANP and NO. Atrial natriuretic peptide (ANP) binds to its receptor (ANP-R), which consists of an integral complex with a particulate guanylate cyclase (GC). Activation of the ANP-R causes hydrolysis of GTP to cGMP. The soluble GC is activated by nitric oxide (NO) coming from extracellular sources or generated by the constitutive Ca^{2+}-dependent NO synthase, present in endothelial cells.

Similar to cAMP is the cGMP concentration regulated by PDE (PDE type V specifically degrades cGMP). Inhibition of PDE activity will reduce cGMP degradation, for instance by zaprinast (inhibits type V), theophylline or 2-isobutyl-1-methyl xanthine (non specific PDE inhibitors). Three types of intracellular cGMP receptor proteins are now known: cGMP-

regulated cation channels (in the retinal rod outer segment), cGMP-stimulated and cGMP-inhibited PDE and cGMP-dependent protein kinase (cGMP-PK). cGMP-PK exists as dimers of 76 kDa (type Iα) and 78 kDa (Iß), and as a monomer of 80 kDa (type II, exclusively found in the intestinal epithelial cells). Discrimination between cGMP-PK type Iα and ß is difficult and both types are often isolated as a mixture in, in particular, smooth muscle cells, platelets and cerebellum.[7,42,94] cGMP-PK can also be activated by cell membrane-permeable agonists, such as 8-bromo-cGMP and the more PDE-resistant 8-chlorophenylthio-cGMP (8-pCPT-cGMP), and by cAMP,[43] dependent on the phosphorylated state of the protein kinase. cGMP-PK activation in smooth muscle cells and platelets is associated with activation of Ca^{2+}-ATPase pumps and Na^{+}/Ca^{2+} exchange and with inhibition of IP_3 formation, thus reducing $[Ca^{2+}]_i$,[8,18,70,91,99] and may therefore be of interest in the regulation of an (Ca^{2+} ion-involved) increase in endothelial permeability. Several studies have suggested a beneficial effect of cGMP and nitric oxide on the endothelial barrier, whereas others claimed the opposite.[3,45,46,80] The effect of cGMP and cGMP protein receptors on the endothelial permeability is one of the major subjects in this thesis.

Activation of the serine/threonine protein kinase C (PKC) may, according to several studies, lead to a reduced endothelial barrier function. This is mainly based on activation studies with phorbol esters and PKC inhibitors.[6,49,56,60,62] At least four PKC subtypes are present in ECs: the Ca^{2+}-dependent PKC α and ß and the Ca^{2+}-independent PKC ε and ζ.[51] The existence of several isoenzymes in one cell type suggests that distinct PKC isoenzymes may activate different cellular pathways and have different protein substrates. A specification of PKC subtype functions will therefore be necessary in the future.

Tyrosine phosphorylation is associated with growth factor receptors, of which the tyrosine residues are intrinsically phosphorylated upon activation. However, tyrosine kinases also phosphorylate cytoskeletal proteins (vinculin, α-actinin), which may affect the endothelial barrier. Although the involvement of tyrosine phosphorylation in the regulation of smooth muscle cell contraction has become clear during recent years,[79] the contribution of tyrosine kinases to the endothelial barrier function has to be determined.

1.7. Aim of this study

The goal of this study is to investigate by which mechanisms the barrier function of cultured endothelial cells, derived from different types of human blood vessels, is improved. From the information given in the foregoing introduction, there is a good reason to believe that determination of the intracellular mechanisms, which are involved in the endothelial permeability *in vitro*, provides information about the regulation of the barrier function of endothelial cells *in vivo*. Knowledge of these mechanisms will help to prevent vascular leakage and will indicate treatments to improve endothelial barrier function in those acute and chronic disease states, in which endothelial permeability becomes disturbed. Furthermore, knowledge of possible differences in barrier function of endothelial cells of various blood vessels and vascular beds may be helpful to understand why local differences (pulmonary vs. peripheral; postcapillary venules vs. larger arteries and veins) in the regulation of endothelial permeability occur, and how they can be influenced.

This study evaluates the effects on intracellular signal transduction pathways and the consequences for the endothelial permeability, triggered by vasoactive substances, directly acting on endothelial cells. In particular endothelial contraction, leading to intercellular gap formation and subsequently vascular leakage, and the intracellular signal molecules and proteins that underlie the prevention or promotion of this inflammatory process were studied. The role of intracellular signal molecules (cAMP, cGMP, calcium ions and nitric oxide), as well as enzymes (cAMP- and cGMP-dependent protein kinases, protein kinase C and phospodiesterase III) and target proteins (actin, myosin and vasodilator-stimulated phosphoprotein) was determined.

- Intracellular cAMP elevation is associated with an improvement of the barrier function, as was determined in cultures of endothelial cells derived from large vessels (aorta, pulmonary artery, umbilical vein and artery). However vascular leakage *in vivo* occurs predominantly in the microvascular bed. Therefore, human foreskin microvascular ECs were cultured and tested for appropriate features, such as barrier characteristics. Then ß-adrenergic stimuli were added to evaluate whether cAMP levels are elevated by these agents (epinephrine, norepinephrine, isoproterenol and salbutamol) and whether they affect endothelial permeability (Chapter 2).
- Interestingly, the effects of atrial natriuretic peptide (ANP) on the pulmonary and systemic vasculature are opposite. Extravasation of fluid and proteins is reduced by the hormone in the former, but is increased in the latter. Because ANP induces intracellular cGMP generation in ECs, the role of ANP and cGMP in permeability of EC monolayers, derived from different origin (umbilical artery and pulmonary artery) was determined (Chapter 3).

- Nitric oxide (NO) may mimick the effects of cGMP on the endothelial permeability, because NO activates cGMP generation in ECs. Therefore, the effects of a NO inhibitor (L-NAME) and NO donor (sodium nitroprusside) were studied (Chapter 3 and 4).
- cGMP mediates its effects via cGMP-activated and cGMP-inhibited enzymes. cGMP-dependent protein kinase (cGMP-PK) may be one of them. However, several studies failed to demonstrate the presence and activity of cGMP-PK in ECs. This was verified in various human EC types by immunocytochemical staining of cGMP-PK by specific antibodies and by using the specific cGMP-PK-activator pCPT-cGMP. cGMP may indirectly affect the cAMP concentration by increasing or decreasing the degradation of cAMP by phosphodiesterases (PDE). The presence and activity of cGMP-inhibited-PDE is determined by specific cGMP-i-PDE inhibitors (Chapter 4 and 5).
- Studies with non-human EC cultures suggested that protein kinase C (PKC) activation is involved in elevated endothelial permeability. PMA and other PKC activators were used to determine the role of PKC in the permeability of human EC monolayers and possible intracellular pathways (Chapter 6).
- The consequences of the findings in this study for prevention of vascular leakage are discussed (Chapter 7).

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CHAPTER 2

MOLECULAR SIEVING CHARACTERISTICS AND β-ADRENERGIC REGULATION OF THE PERMEABILITY OF MONOLAYERS OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS *IN VITRO*.

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ABSTRACT

Human foreskin microvascular endothelial cells (MVEC) were cultured and characterized to evaluate the effect of adrenergic stimulation on the endothelial barrier function. The MVEC formed tight monolayers with a permeability coefficient (PC) of 6.6 $cm.s^{-1}.10^{7}$ for 38.9 kDa dextran and a transendothelial electrical resistance (TEER) of 13.5 $\Omega.cm^2$. Addition of thrombin (1U/ml) strongly increased the permeability, reduced the TEER and induced actin-myosin association, as determined by immunocytochemistry. Forskolin prevented thrombin-induced effects. The MVEC monolayers formed a selective barrier to (macro)molecules with Stokes radii ranging from 4.4 to 162 Å (180 to 487,000 Da), similar to human aorta and umbilical vein EC monolayers. The molecular sieving characteristics displayed a biphasic course suggesting the existence of two populations of differently sized pores. Epinephrine and norepinephrine reduced the endothelial permeability albeit at rather high concentrations (10 to 100 nM), which was attended by an increase in the intracellular cAMP concentration. The ß-adrenergic receptor antagonist propranolol prevented this effect, in contrast to the α1-receptor antagonist corynanthine. The α1-adrenergic agonist phenylephrine and α2-adrenergic agonist clonidine had no effect on the MVEC barrier. In contrast clonidine increased the permeability of aorta and umbilical vein EC monolayers. The ß-adrenergic agonists isoproterenol and salbutamol reduced the endothelial permeability and increased the cAMP concentration in all endothelial cell types. The thrombin-induced increase in permeability was strongly reduced by the catecholamines and β-adrenergic agonists. Prolonged incubation with ß-adrenergic agonists reduced the responsiveness of the endothelial cells to adrenergic stimulation. These data provide barrier characteristics of human MVEC monolayers in vitro. They indicate that the permeability of MVEC monolayers is reduced by ß2-adrenergic stimulation, and that desensitization of this response occurs after prolonged incubation with a ß-adrenergic agonist.

INTRODUCTION

The endothelium, which forms the inner lining of blood vessels, is a selective barrier to blood constituents. Contraction of endothelial cells (EC) of, in particular, postcapillary venules during inflammation or by damage to the EC monolayer, however, leads to extravasation of fluid and macromolecules, and may cause life threatening edema. It is therefore important to determine whether vascular leakage can be prevented or diminished. Bradykinin or histamine-induced plasma protein leakage of guinea pig pulmonary microvessels was prevented by the ß2-adrenergic stimulus terbutaline.[19] It has been proposed that this was due to a direct effect on the endothelial cells. Adkins and colleagues[1] suggested from a study in rabbits, that prevention by isoproterenol of ischemic-reperfusion-induced increase in pulmonary capillary permeability, might be caused by a reduction in the size of endothelial intercellular junctions. Additionally, patients with systemic capillary leak syndrome were successfully treated with terbutaline, which reduced the attacks of hypovolaemic shock, although only for a limited period.[15,18] Thus, ß-adrenergic stimulation seemed to have beneficial effects on the endothelial barrier.

Endothelial cells in culture can be helpful for the determination of the endothelial response to adrenergic stimulation. ß-adrenergic receptors on EC have already been identified on bovine aorta EC.[45] Furthermore, stimulation by isoproterenol,[12,32] norepinephrine[6,29] or formoterol[56] in *in vitro* models indicated that endothelial cells respond to ß-adrenergic agonists. A disadvantage of these models is, however, the relatively high permeability of monolayers derived from macrovessels which often have permeabilities for macromolecules that are several orders of magnitude higher than found in *in vivo* studies.[2,8,39,44,56] Because (inflammation-induced) endothelial leakage occurs mainly in the microvasculature,[31,47] we have set up a study to extend the earlier findings to human microvascular EC. The sieving characteristics of monolayers of human EC isolated from the foreskin microvasculature were determined and recognized as tight and highly selective. The EC monolayers were stimulated by the physiological adrenergic stimuli epinephrine and norepinephrine, as well as specific α1-, α2- and ß agonists. ß2-adrenergic stimulation strongly attenuated the permeability of macromolecules through the microvascular EC monolayers. Prolonged ß-adrenergic stimulation caused desensitization of the cells to ß-adrenergic stimuli.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics from Corning (Corning, NY, USA) or Costar (Cambridge, MA, USA); and Transwells (diameter 0.65 cm, pore size 3 μm) from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al.[30] Human serum was obtained from the local blood bank and was prepared from fresh blood taken from healthy donors; the sera were pooled, and stored at 4°C. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY, USA) and heat-inactivated before use (30 min, 56°C). Pyrogen-free human serum albumin (HSA) was purchased from the Central Laboratory of Blood Transfusion Service (Amsterdam, the Netherlands). Salbutamol was obtained from Glaxo (Zeist, The Netherlands); Norepinephrine, epinephrine, phenylephrine, clonidine, isoproterenol, propranolol, corynanthine, horseradish peroxidase EC 1.11.1.7 type I (HRP), fluorescein isothiocyanate- or rhodamine-labelled dextrans (dextran-FITC/TRITC) with molecular masses of 4400, 9600, 17200, 35600, 38900, 147800 and 487000 D were obtained from Sigma Chemical Company (St. Louis, MO, USA); [^{14}C]-sucrose and [^{3}H]-galactose from Dupont NEN (Bad Hamburg, Germany); Low density lipoproteins (LDL) were prepared by density gradient ultracentrifugation of human serum according to the method of Redgrave[38] and iodinated with ^{125}INa as described by Bilheimer et al[5]; bovine α-thrombin was obtained from LEO Pharmaceutical Products (Ballerup, Denmark); forskolin from Hoechst (La Jolla, CA, USA); isobutyl-methyl-xanthine (IBMX) from Janssen Chimica (Beerse, Belgium); fura 2-AM from Molecular Probes (Eugene, OR, USA). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR, USA); anti-myosin IgG (non-muscle) from Sanbio (Uden, Netherlands); Rabbit-anti mouse IgG-FITC from Dakopatts (Denmark).

Isolation and culture of endothelial cells

Human endothelial cells (EC) from umbilical vein and aorta were isolated and characterized as described previously.[23,49,50] Human foreskin microvascular endothelial cells (MVEC) were isolated and purified according to the procedures described by Davison et al[10] and Voyta et al.[53] The MVEC were characterized by common EC markers, such as the expression of PECAM-1 (Figure 1a), von Willebrand factor in granules, binding of Ulex europaeus lectin-I and the uptake of DiI-Ac-LDL,[51] and by specific markers PAL-E and α1-integrine, which identify microvascular EC *in vivo* and *in vitro*.[11,41,51] The blood vessels of human origin were obtained according to the guidelines of the Institutional Review Board of the University Hospital Leiden.

Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin, 100 U/ml penicillin and 0.1 mg/ml streptomycin and kept at 37°C under 5% CO_2/95% air. The medium was renewed every other day. Confluent EC monolayers were released with trypsin-EDTA and subcultured on fibronectin-coated dishes, filters or glass-slides. Human foreskin microvascular EC (fifth to eleventh passage), aorta EC (fourth to sixth passage) or umbilical vein EC (first and second passage) were used for the evaluation of the barrier function, immunocytochemistry and cyclic nucleotides and $[Ca^{2+}]_i$ measurements.

Evaluation of the barrier function

Endothelial cells cultured on filters were used 5 days after seeding. Exchange of macromolecules through the endothelial monolayers was investigated by assay of the transfer of galactose, sucrose, horseradish peroxidase (HRP, 5μg/ml), dextran-FITC, dextran-TRITC (1 mg/ml) and low density lipoproteins (LDL) as described previously.[17,27,28,29] Briefly, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) to form a tight monolayer. The cells were incubated in M199 with 1 % albumin one hour before the experiment and throughout its course. The abovementioned tracer molecules were added to the upper compartment of the Transwell™ system in the presence or absence of adrenergic agonists, adrenergic antagonists and/or thrombin. Samples were taken from the lower compartment and an equal volume of Medium 199 with 1% albumin was re-added to this lower compartment. Cells were kept at 37°C under 5% CO_2/95% air. Passage experiments were performed in duplicate or triplicate. The concentration of the tracer molecules in under and upper compartiment were determined as previously described.[17] The permeability coefficient (PC) was derived from Fick's law of diffusion and is determined by:

PC = mass flux tracer molecule / ($[\text{tracer molecule}]_{UC}$-$[\text{tracer molecule}]_{LC}$),

where UC is the upper compartment and LC is the lower compartment. The mass flux is expressed in $ng.cm^{-2}.h^{-1}$.

Measurement of cytoplasmic cyclic nucleotide and calcium ion concentration

The cyclic nucleotide concentrations in human EC were determined by radio-immunoassay as described by Draijer et al.[17] Briefly, to determine the concentrations of cAMP and cGMP the cells were preincubated for 15 min with the phosphodiesterase inhibitor IBMX (1 mM) and subsequently with the concentrations of adrenergic stimulus indicated and, if indicated, 1U/ml thrombin. After 15 min the experiment was terminated by the addition of 3.5% perchloric acid. The cell lysates were neutralized by $KHCO_3$, dried under a stream of N_2-gas and kept at -20°C until determination by radio-immunoassay (Amersham, Amersham, UK).

The cytoplasmic calcium concentration was determined in fura2-AM loaded microvascular endothelial cells on coverslips, as described previously.[17]

Immunocytochemistry

Glass coverslips were coated for 45 min with 1% gelatin which was cross-linked by an additional incubation of 15 min with 0.5% glutaraldehyde. The glass coverslips were 5 times washed with Medium 199. EC were seeded on the glass coverslips and at confluency, if indicated, incubated with 1U/ml thrombin for one hour in medium 199 with 1% albumin, and then washed with medium 199, fixed for 10 min with paraformaldehyde, permeabilized with 0.1% Triton-X100 for 2 min and washed again. The endothelial monolayers were then stained with rhodamine-phalloidin (staining F-actin) or antibodies against PECAM-1 or non-muscle myosin for 30 min, washed three times for 5 min with phosphate buffered saline (PBS) and incubated with a second fluorescent-conjugated antibody. After 30 min the cells were for 5 min three times washed with PBS and embedded in p-phenylenediamine (PPD).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis as indicated in the text was performed with the Wilcoxon's rank sum test. Statistical significance was assumed if $p < 0.05$.

RESULTS

Identification and barrier characteristics of human microvascular EC monolayers.

Human microvascular endothelial cells (MVEC) formed a monolayer of closely apposed cells, as shown by decoration of the cells with antibodies against PECAM-1 (CD31) (Figure 1a). When cultured on porous fibronectin-coated polycarbonate filters, these cells formed a tight monolayer, which potently reduced the passage of a 38.9 kDa dextran (Figure 1b, closed circles). After addition of thrombin the permeability increased (Figure 1b, open circles) and tiny gaps became occasionally visible between the cells (not shown). In agreement with previous studies on umbilical vein and artery endothelial cells,[7,17,23,27] this increase was paralleled by an initial increase in the concentration of cytoplasmic calcium ions (Figure 1b, inset), and with a reduction of the transendothelial electrical resistance (from 13.5 $\pm$ 1.4 to 5.4 $\pm$ 0.7 Ohm.cm^2 after 10 min incubation with 1U/ml thrombin; mean $\pm$ SEM of 12 cultures). Elevation of the cellular cAMP concentration in the cells by the adenylate cyclase activator forskolin prevented the thrombin-induced increase in permeability (Figure 1b, squares) and largely restored the electrical resistance after exposure to 1U/ml thrombin (to 10.2 $\pm$ 0.7 Ohm.cm^2, 5 cultures). In thrombin-exposed MVEC a close association between actin filaments and non-muscle myosin was visible, e.g. in stress fibres (Figure 1c: actin; 1d: myosin). After exposure of the cells to forskolin, this interaction disappeared and non-muscle myosin was found dispersed in the cytoplasm (Figure 1e: actin; 1f: myosin).

When tracer molecules (mainly dextrans) with different Stokes radii (range 4.4 to 162 Å, corresponding to molecular masses of 180 to 487,000 Da) were used for the determination of endothelial permeability, clear molecular sieving characteristics were observed under basal conditions (Figure 2a). The biphasic aspect of the curve indicates that the different passage rates at various molecular masses are not exclusively related to differences in diffusion rate, but rather reflect the passage of molecules through small and large pores. Comparison of the permeability of MVEC with that of human aorta EC and human umbilical vein EC *in vitro* demonstrated that the molecular sieving characteristics of the three types of endothelial monolayers in culture are largely comparable (Figure 2a-c).

When the permeability of human endothelial monolayers was increased by addition of thrombin, the molecular sieving properties of the monolayers changed considerably (Figure 2a-c, insets). In particular the permeability for large molecules increased markedly. This further indicates that the molecular sieving characteristics were specific and not primarily due to differences in the diffusion rate of the agents with different molecular masses.

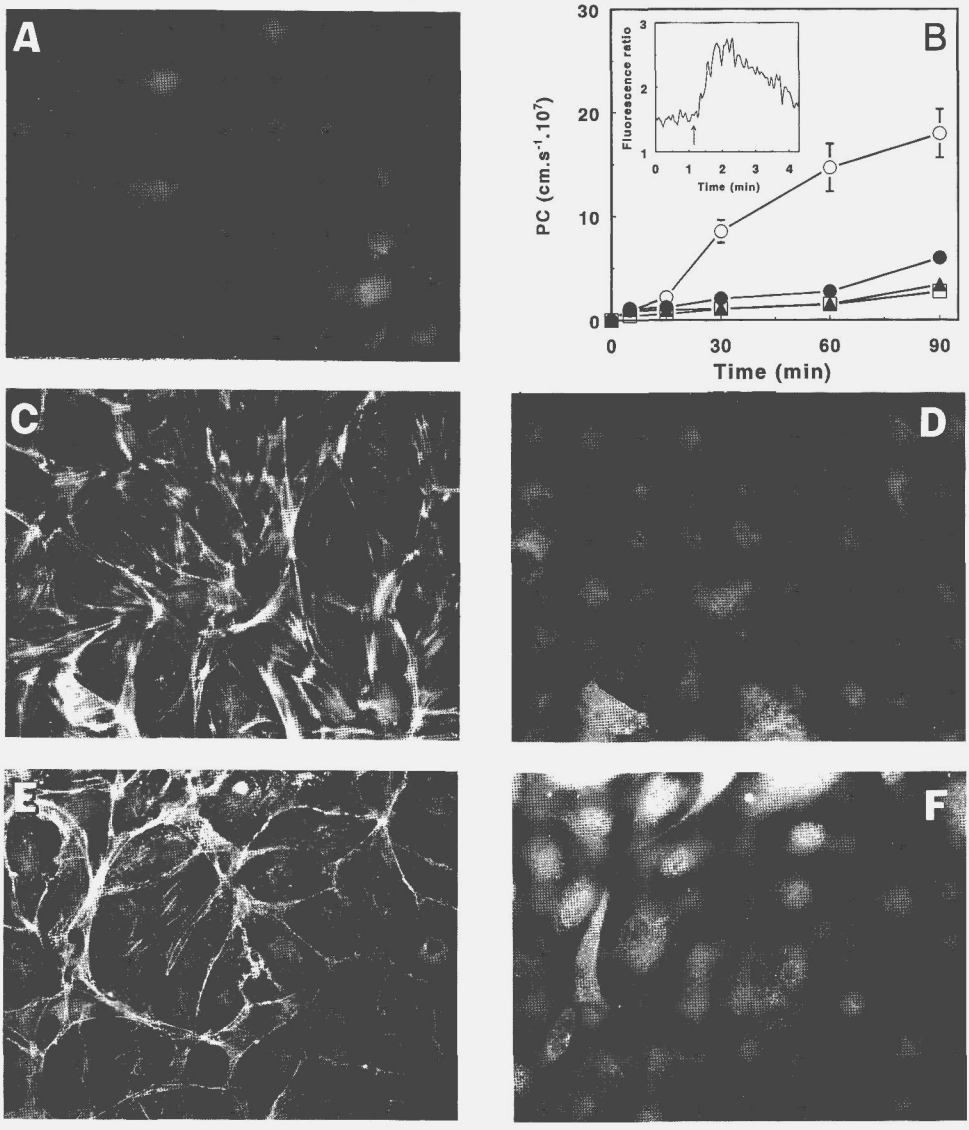

Figure 1: Characterization of human microvascular EC monolayers under basal and thrombin-stimulated conditions. A: The cells formed tight monolayers and stained positive for the specific EC marker PECAM-1. B: The basal permeability (●) of a 38,9 kDa dextran through the EC monolayers was increased by addition at t=0 of thrombin (1 U/ml, ○). Preincubation with forskolin (10μM) for 5 min improved the barrier function under basal (▲) and thrombin-stimulated (□) conditions. B inset: The intracellular calcium ion concentration indicated by the fluorescence ratio of 340/380 nm excitation wavelenght of fura-2 loaded EC increased immediately after addition of thrombin (arrow). C-F: Double immunostaining of actin and non-muscle myosin (Fig C and E actin; Fig D and F myosin). Endothelial cells were incubated with 1 U/ml thrombin for one hour in the absence (C, D) or presence (E, F) of 10 μM forskolin. The cells were fixed with paraformaldehyde and stained with rhodamine-phalloidine and an anti-myosin monoclonal antibody as described in the Methods section.

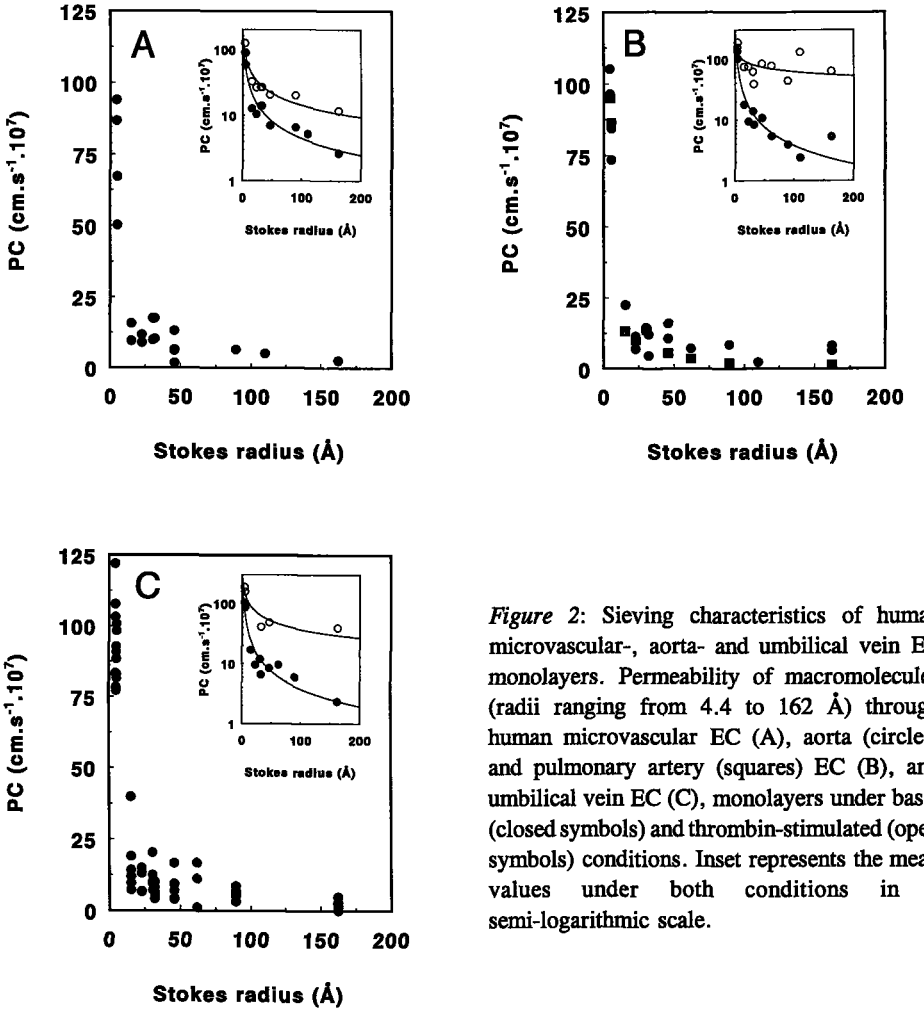

Figure 2: Sieving characteristics of human microvascular-, aorta- and umbilical vein EC monolayers. Permeability of macromolecules (radii ranging from 4.4 to 162 Å) through human microvascular EC (A), aorta (circles) and pulmonary artery (squares) EC (B), and umbilical vein EC (C), monolayers under basal (closed symbols) and thrombin-stimulated (open symbols) conditions. Inset represents the mean values under both conditions in a semi-logarithmic scale.

Effect of adrenergic stimulation on permeability and cyclic nucleotide concentration of human microvascular endothelial cells.

Incubation of MVEC with the physiological catecholamines epinephrine or norepinephrine reduced the endothelial permeability in a concentration-dependent way under basal conditions (Figure 3a,b), and counteracted the increase in permeability induced by thrombin (Figure 3c,d). These effects were prevented by a 15 min preincubation of the cells with the β-adrenergic antagonist propranolol, but not with the α1-adrenergic antagonist corynanthine. Fairly high concentrations of epinephrine and norepinephrine were needed

(10^{-8}-10^{-7} M) to obtain an effect on basal endothelial permeability *in vitro*. Estimation of the cellular cyclic AMP concentration in these cells indicated that indeed these concentrations were needed to stimulate the β-adrenergic receptor-coupled adenylate cyclase (Figure 3a,b insets).

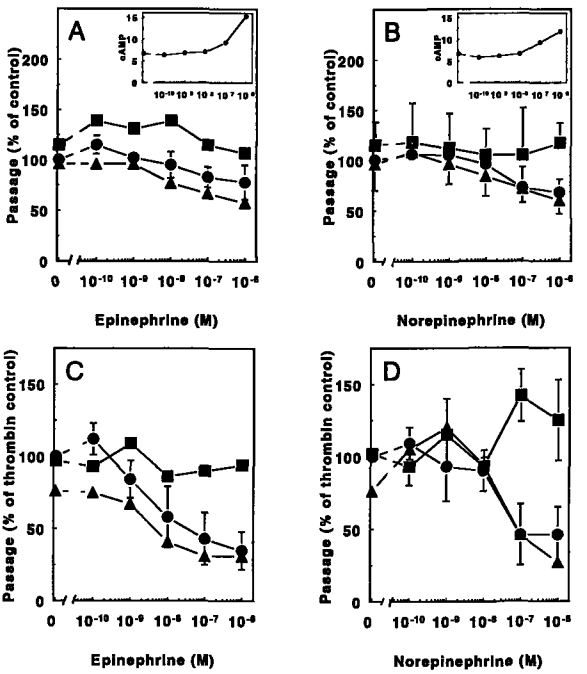

Figure 3: Effect of epinephrine and norepinephrine on the basal (A, B) and 1U/ml thrombin-stimulated (C, D) permeability of a 38.9 kDa dextran through human microvascular EC monolayers. The effect of (nor)epinephrine on the passage of FITC-dextran was measured after one hour (●). This effect was prevented by a 15 min preincubation with propranolol (1 μM, ■), but not by corynanthine (1 μM, ▲). The intracellular cAMP concentration (in pmol/3.5x10^5 cells) increased concomitantly (insets A and B). Data represent mean ± SEM (range) of two or three experiments.

Because norepinephrine and epinephrine activate both α and β adrenoceptors and these adrenoceptors may counteract each other, we used more selective agonists. The β-adrenergic agonists isoproteronol (β) and salbutamol (β2) reduced endothelial permeability (Figure 4a) and enhanced the cAMP concentration of MVEC (as depicted for isoproterenol in Figure 5a), thus mimicking the effects of epinephrine and norepinephrine. However, the α1-adrenergic agonist phenylephrine also reduced the permeability at high concentrations (Figure 4a). This was due to cross reactivity with the β-adrenoceptor, because at these high concentrations

(10^{-5} M) phenylephrine caused elevation of cAMP (Figure 5a) and the permeability reducing effect of phenylephrine was inhibited by propranolol (not shown).

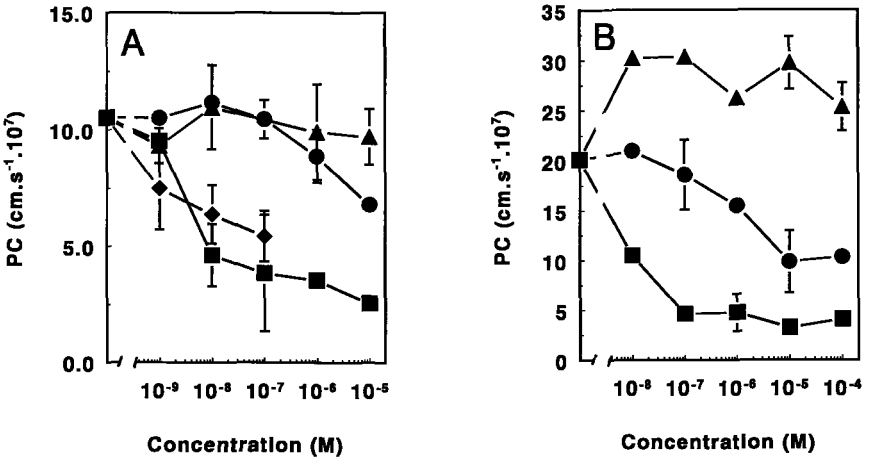

Figure 4: Effect of phenylephrine, clonidine, isoproterenol and salbutamol on the permeability of a 38,9 kDa dextran through EC monolayers. A: The permeability of human microvascular EC monolayers was measured one hour after addition of phenylephrine (●), clonidine (▲), isoproterenol (■) or salbutamol (◆). B: similar to Figure 4A for human aorta EC monolayers. Data represent mean ± range of a representative experiment.

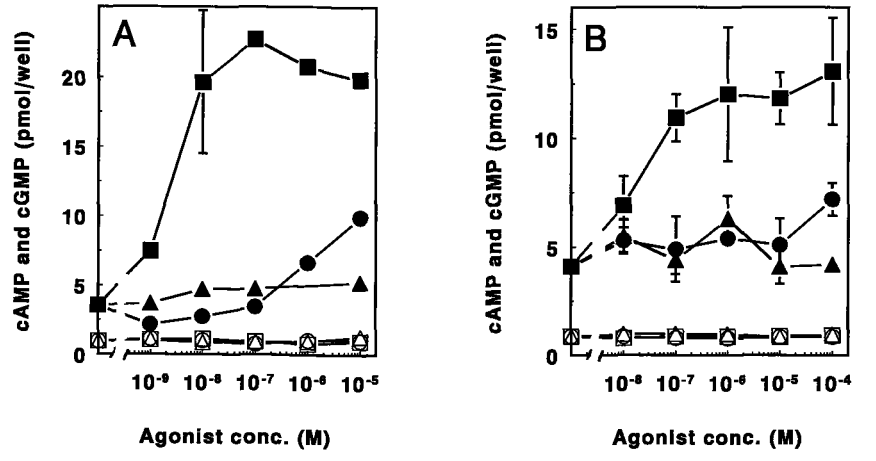

Figure 5: A: Intracellular cAMP (filled symbols) and cGMP (open symbols) concentrations (pmol/3.5x10^5 cells) in human microvascular EC after 15 min incubation with phenylephrine (circles), clonidine (triangles) or isoproterenol (squares). B: similar to figure 5A for aorta EC. Data represent mean ± range of a representative experiment.

Isoproterenol had a similar effect in human aorta EC (Figure 4b and 5b) and umbilical vein EC (Table 1 and 2). The β-adrenergic stimulation by isoproterenol improved the barrier function of MVEC and aorta EC with a half-maximal effective concentration (EC_{50}) of 4 and 18 nM, respectively. The EC_{50} values of the elevation of the cellular cAMP concentration by isoproterenol were comparable, being 2.2 and 25 nM, respectively. Apparently human aorta and umbilical vein EC contained more active α2-adrenoceptors than MVEC, because clonidine tended to enhance endothelial permeability in aorta and umbilical vein EC (Figure 4b; Table 1), whereas it was relatively ineffective in MVEC (Figure 4a; Table 1).

The cGMP concentration was not affected by the adrenergic stimuli irrespective of the EC type.

Table 1: Passage of a 38.9 kDa dextran through monolayers of human microvascular, aorta and umbilical vein EC in the absence or presence of phenylephrine (10μM), clonidine (10μM) or isoproterenol (10μM).

	Passage of dextran ($cm.s^{-1}.10^7$)		
Addition	Microvascular EC	Aorta EC	Umbilical vein EC
None	6.6 ± 2.4 (10)	14.4 ± 5.9 (6)	8.6 ± 2.0 (10)
Phenylephrine	5.8 ± 1.0 (8)	19.3 ± 5.0 (6)	9.0 ± 1.1 (10)
Clonidine	6.5 ± 1.0 (7)	15.6 ± 1.5 (5)	8.6 ± 1.0 (8)
Isoproterenol	3.3 ± 0.8* (8)	4.3 ± 0.8* (5)	6.8 ± 1.1* (10)
Thrombin	57.1 ± 19.9 (9)	77.1 ± 3.3 (2)	59.3 ± 19.0 (7)
+Phenylephrine	32.3 ± 9.0*(7)	75.8 ± 1.3 (2)	44.8 ± 7.4 (7)
+Clonidine	51.6 ± 14.6 (7)	126 ± 7.1 (2)	75.1 ± 5.6 (5)
+Isoproterenol	22.9 ± 6.1*(8)	25.4 ± 4.5 (2)	41.9 ± 6.8*(7)

The passage of dextran in one hour was measured under basal and thrombin-stimulated (1 U/ml) condition. Values are mean ± SEM of the number of different cultures (n). *$P < 0.05$ vs no addition of catecholamine.

Table 2: Cyclic AMP and cyclic GMP concentration in microvascular, aorta and umbilical vein EC monolayers in the absence or presence of phenylephrine (10μM), clonidine (10μM) or isoproterenol (10μM).

	Cyclic nucleotide concentration (pmol/3.5x10^5 cells)		
	Microvascular EC	Aorta EC	Umbilical vein EC
cAMP:			
Control	7.4 ± 2.0 (5)	6.5 ± 1.8 (4)	4.1 ± 0.7 (10)
Phenylephrine	10.1 ± 1.9* (5)	8.6 ± 2.3 (4)	5.3 ± 1.1*(10)
Clonidine	7.7 ± 2.0 (5)	6.9 ± 1.9 (4)	4.0 ± 1.1 (7)
Isoproterenol	17.0 ± 4.2* (5)	17.9 ± 4.9 (4)	5.4 ± 0.6*(10)
cGMP:			
Control	1.6 ± 0.3 (4)	1.7 ± 0.4 (3)	0.7 ± 0.2 (8)
Phenylephrine	1.7 ± 0.4 (4)	1.6 ± 0.4 (3)	0.7 ± 0.1 (8)
Clonidine	1.5 ± 0.2 (4)	1.7 ± 0.5 (3)	0.5 ± 0.1 (5)
Isoproterenol	1.3 ± 0.3 (4)	1.5 ± 0.8 (3)	0.9 ± 0.2 (8)

The cells were preincubated for 15 min with IBMX (1mM). The cyclic nucleotides concentration was determined 15 min after the addition of the stimuli. Values are mean ± SEM of the number of different cultures (n). *$P<0.05$ vs corresponding control value.

Desensitization of ß adrenoceptors

Because stimulation of ß adrenoceptors improves the barrier function, we wondered whether this effect would be maintained after chronic ß adrenergic stimulation. Initially, the ß2 selective agonist salbutamol was used, which decreased basal and thrombin-stimulated endothelial permeability (Table 3). Preincubation of microvascular EC with salbutamol (100 nM) for 24 hours reduced the improvement of the endothelial barrier by salbutamol itself (Table 3). Similarly, endothelial cells cultured for 24 hours in the presence of epinephrine or isoproterenol caused a complete desensitization of the cells towards the same stimulus (Figure 6a,b). Apparently the preincubation caused a downregulation of ß2-receptors on the endothelial cells. Preincubation of the cells with isoproterenol for 24 hours caused a complete desensitization for isoproterenol, epinephrine and salbutamol (Figure 6c).

Table 3: Desensitization of human microvascular EC monolayers to the ß2-agonist salbutamol by chronic ß-adrenergic stimulation.

	Passage of dextran ($cm.s^{-1}.10^7$)			
Addition	control preincubation		24 h preincubation with salbutamol	
None	1.8 ± 0.3	(100%)	2.1 ± 0.4	(124 ± 7%)
Salbutamol	1.1 ± 0.3*	(67 ± 10%)	1.4 ± 0.3	(94 ± 14%)
Thrombin	29.3 ± 8.0	(100%)	32.9 ± 7.1	(156 ± 36%)
Thrombin+ Salbutamol	13.0 ± 3.0*	(54 ± 7%)	26.8 ± 8.9	(114 ± 27%)

The passage of a 38.9 kDa dextran through microvascular EC monolayers in the absence or presence of salbutamol (100nM) or thrombin (1U/ml) was measured. Before the start of the experiment the cells were preincubated in serum-containing medium supplemented with 100 nM salbutamol or without it (control). Subsequently, the medium was replaced by M199 medium + 1% albumin or the same medium supplemented with 100 nM salbutamol, and FITC-labeled dextran was added to the upper compartment. After 2 h incubation 1 U/mL thrombin was added. The passage of the dextran was determined under basal and thrombin-stimulated condition after an one hour period. Values are mean ± SEM of 5 to 7 different cultures; the percentages of the corresponding control value are given in parentheses. *$P < 0.05$ vs corresponding control value.

DISCUSSION

This study describes the barrier properties of human microvascular endothelial cell monolayers *in vitro* and their molecular sieving characteristics. ß-adrenergic stimulation reduces the microvascular endothelial permeability, but desensitization occurs after prolonged stimulation.

We demonstrate for the first time that human microvascular EC form in culture a tight monolayer with barrier-selectivity towards differently sized macromolecules (Fig 2a). The curve obtained by comparing the permeability of dextrans with different molecular masses fits with the two-pores model,[35,39] where free diffusion of molecules is restricted by endothelial cell junctions containing many small and a few large pores. The curves suggest that, in contrast to larger molecules, the diffusion rate of small molecules such as galactose and sucrose is not restricted by pores with a radius of approximately 15 Å. Diffusion of large molecules through the endothelial monolayer may be due to openings larger than 200 Å. Possibly, plasmalemmal vesicles represent these large pores.[36] In rat cardiac capillaries such

a restriction was also observed: the passage of macromolecules larger than horseradish peroxidase (radius 30 Å) was strongly restricted.[54] The endothelial permeability to macromolecules in our *in vitro* model approaches that of capillaries of frog mesentery *in vivo*,[8] but still leaks at least 10 times more than continuous endothelium from skeletal and cardiac muscle and from alveolar capillaries.[39] Nevertheless, the permeability of the monolayers responded clearly to thrombin by a significant increase in the passage of (macro)molecules, a considerable loss of molecular sieving characteristics and a reduction of the trans-endothelial electrical resistance. These data agrees with previous data on endothelial cells cultured from large vessels and reflects the increased interaction of actin and myosin, as suggested by the thrombin-induced actin-myosin colocalization (Fig 1c,d), and subsequent enlargement of intercellular spaces. This effect was counteracted by forskolin, which elevates the cellular cAMP level, similarly to that found in human umbilical vein EC.[7,29] In the latter cells, it has been shown that this is accompanied by a reduced phosphorylation of the myosin light chain.[20,33] Phosphorylation of myosin light chain is required for effective actin-myosin interaction (notice loss of actin-myosin colocalization in the presence of forskolin, Fig 1e,f).[42,55]

Evaluation of the effects of natural adrenergic stimuli on the permeability of the microvascular monolayers revealed that epinephrine and norepinephrine had a rather small effect on endothelial permeability under basal permeability conditions. Submicromolar concentrations of norepinephrine and epinephrine were needed to induce a small decrease in endothelial permeability, which is comparable with the effects of norepinephrine on bovine aorta ECs.[6,56] Because similar concentrations were needed to induce a moderate increase in intracellular cAMP level, this suggested that the adrenoceptor-coupled adenylate cyclase is activated at rather high concentrations of the catecholamines, which may be due to low ß-adrenoreceptor affinity. Alternatively, homologous desensitization of the ß-adrenoceptor by the ß-adrenergic receptor kinase (ß-ARK) may have caused a reduction in the coupling of receptor and adenylate cyclase.[37] However, one would expect then that similar high concentrations of isoproterenol would be needed to increase cAMP levels and to decrease the endothelial permeability. This was not the case (Fig 4 and 5). The thrombin-induced increase in permeability was reduced by epinephrine and norepinephrine at concentrations comparable to that found under basal conditions (EC_{50} 3 and 30 nM, respectively), but the reduction was much stronger in stimulated cells. This is expected, because elevation of cAMP reduces the phosphorylation of the myosin light chain, thus preventing actin-myosin interaction.[20,33] Via this and possibly additional mechanisms β-adrenergic stimulation of the adenylate cyclase prevents the contraction of endothelial cells.

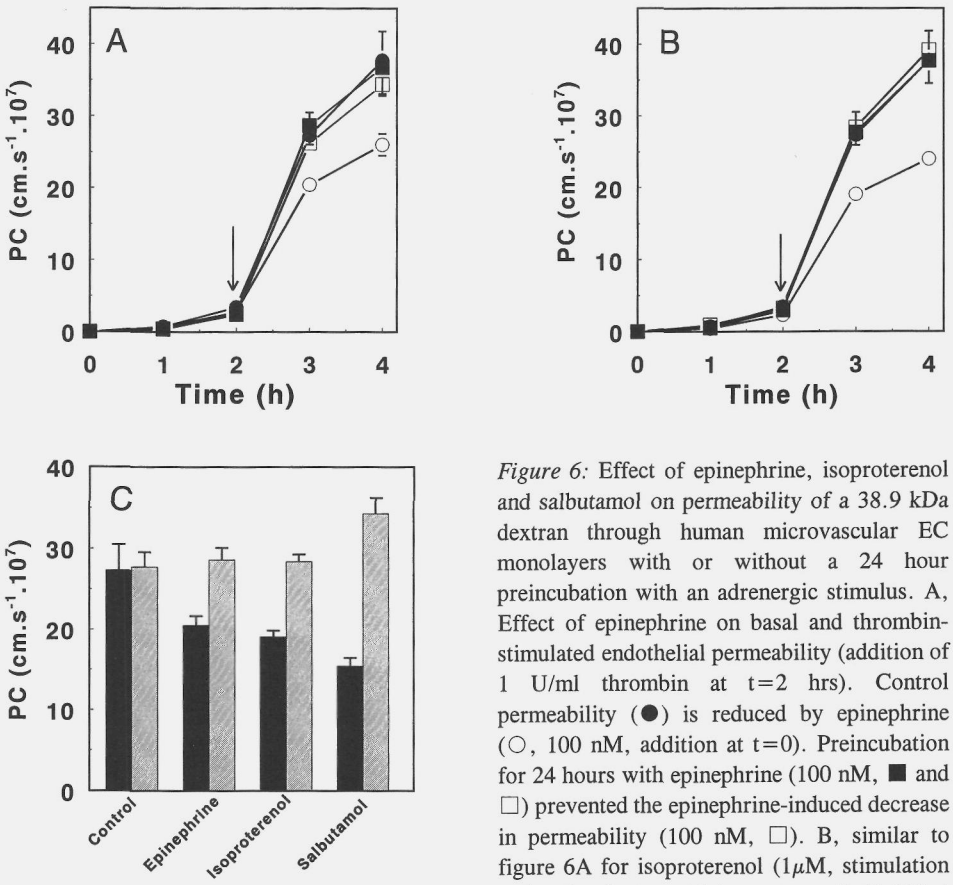

Figure 6: Effect of epinephrine, isoproterenol and salbutamol on permeability of a 38.9 kDa dextran through human microvascular EC monolayers with or without a 24 hour preincubation with an adrenergic stimulus. A, Effect of epinephrine on basal and thrombin-stimulated endothelial permeability (addition of 1 U/ml thrombin at t=2 hrs). Control permeability (●) is reduced by epinephrine (○, 100 nM, addition at t=0). Preincubation for 24 hours with epinephrine (100 nM, ■ and □) prevented the epinephrine-induced decrease in permeability (100 nM, □). B, similar to figure 6A for isoproterenol (1μM, stimulation and preincubation with isoproterenol). C, Effect of epinephrine (100 nM), isoproterenol (100 nM) and salbutamol (100 nM) on thrombin-enhanced endothelial permeability after a 24 hours preincubation with isoproterenol (1 μM) (hatched bars) or without it (filled bars). The thrombin-enhanced permeability was determined after 1 hour. Data represent mean ± SEM of triplicate determinations.

The endothelial barrier function of microvascular EC *in vitro* was not significantly affected by α1- or α2-adrenergic stimulation by phenylephrine and clonidine, respectively. Phenylephrine reduced the endothelial permeability at micromolar concentrations, but this

was due to stimulation of ß-adrenoceptors. Several studies have indicated that arterial EC express α2-adrenoceptors and that their activation causes a rise in the cytoplasmic calcium ion concentration and stimulates the release of nitric oxide.[3,24,34] Whereas the α2-adrenoceptor agonist clonidine induced an increase in the permeability of human aorta EC monolayers indicating the presence of α2-receptors on these cells *in vitro*, similarly as previously shown on umbilical artery EC,[29] we did not find an indication for α2-adrenoceptor activity on microvascular EC (Figure 5; Table 2). Whether α2-expression is preferentially or exclusively expressed in EC of muscular blood vessels remains to be determined.

A number of studies demonstrated that exogenously added catecholamines or β-adrenergic agonists reduce vascular leakage after stimulation of the endothelium by vasoactive substances in vivo.[4,13,14,21,22,25,26,46,56] These agents may act both directly on the barrier function of the endothelium or indirectly via activation of leukocytes adhered to the endothelium. Extravasation of red blood cells in thrombocytopenic hamsters was reported to be largely prevented by norepinephrine,[43] as was polymorphonuclear leukocyte diapedesis through bovine aorta EC monolayers by the same agonist.[16] Our data show that the β-agonist isoproterenol and the ß2-agonist salbutamol reduced the permeability of microvascular EC monolayers with an equal potency indicating a direct effect of these agents on the endothelial barrier function. They agree with previous data on experimental animals indicating that stimulation of particularly the ß2-subtype of adrenoceptors, is important.[46,56] In a recent histochemical study on rat trachea Baluk and McDonald[4] demonstrated that β2-adrenoceptor stimulation reduces macrovascular leakage by inhibiting intercellular gap formation, rather than by increasing the gap size. This further stresses the direct effect of catecholamines on endothelial barrier function. Although a role of catecholamines in maintaining the endothelial barrier function appears evident in conditions in which the endothelial permeability is enhanced by exposure of the endothelium to vasoactive substances, their possible role in maintenance of the endothelial barrier function under basal conditions remains still to be established. Furthermore, the endothelial response to catecholamines may differ in various parts of the vascular bed and may alter when the endothelial functioning is impaired, such as in atherosclerotic vessels: these vessels display an increased sensitivity to the constrictor effect of catecholamines.[52]

Preincubation for 24 hours with salbutamol reduced the decrease by salbutamol and other β-adrenergic stimuli of both basal and thrombin-induced increase in endothelial permeability (Table 3; Figure 6). Several observations suggest that the ß2-adrenoceptor desensitization of endothelial cells in our *in vitro* model also can occur *in vivo*. Patients which were treated with β-adrenergic agents to prevent premature delivery occasionally develop pulmonary edema.[9,40,48] Furthermore, treatment with terbutaline of incidental patients with capillary

leakage syndrome was only initially effective, probably because of adaptation of the vascular tree to the drug.[15,18]

In conclusion, our data indicate that human MVEC in culture can be used to address specific questions regarding to endothelial barrier function. $ß_2$-adrenergic stimuli reduce the permeability of thrombin-stimulated human microvascular EC in vitro. Chronic stimulation with β-adrenergic agonists desensitizes the cells towards these agents. Therefore, it is worthwhile to evaluate in future studies whether microvascular leakage may become increased during frequent treatment with ß2-adrenergic stimuli, because of tachyphylaxis of EC.

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CHAPTER 3

CYCLIC GMP MEDIATED DECREASE IN PERMEABILITY OF HUMAN UMBILICAL AND PULMONARY ARTERY ENDOTHELIAL CELL MONOLAYERS

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ABSTRACT

Endothelial cell contraction plays a pivotal role in the increased extravasation of fluid and macromolecules in vascular leakage. Previous studies have indicated that elevation of the adenosine 3',5'-cyclic monophosphate (cAMP) concentration can improve the endothelial barrier function. In analogy with smooth muscle cell contraction, which is inhibited by both cAMP and guanosine 3',5'-cyclic monophosphate (cGMP), we have compared the role of cAMP and cGMP in the regulation of the permeability of human endothelial cell monolayers. The cellular cGMP concentration was elevated three- to five-fold after addition of 10^{-7} M atrial natriuretic peptide (ANP) or 10^{-4} M sodium nitroprusside (SNP), both under basal and thrombin-stimulated conditions. After exposure to thrombin, cGMP generation by ANP or SNP or addition of 8-bromo-cGMP significantly suppressed the increase in permeability. Inhibition of nitric oxide production with 10^{-4} M N^G-L-nitro-arginine methyl ester increased the permeability of endothelial monolayers in the majority of the tested cultures, an effect that could be counteracted by addition of 8-bromo-cGMP or ANP. An increase of cAMP upon the addition of forskolin reduced the permeability in all endothelial cell strains under basal conditions and after exposure to thrombin. The forskolin- and 8-bromo-cGMP-mediated decreases in permeability were attended by increases in transendothelial electrical resistance. These *in vitro* data indicate that, in addition to cAMP, cGMP can act as a potent fine-regulator of endothelial permeability.

INTRODUCTION

Tight intercellular junctions are essential for the barrier function of the vascular endothelium. Widening of the intercellular clefts in postcapillary venules augments the permeation of fluids and macromolecules across the vessel wall, leading to interstitial edema. Early studies on the effect of histamine on vascular leakage suggested that intercellular gaps occur by endothelial cell contraction (1). Nowadays it has become clear that the endothelial actomyosin system plays a central role in the regulation of endothelial permeability (2). Analogous to smooth muscle cell contraction, myosin light-chain phosphorylation directs the actin-myosin based contraction in endothelial cells, a process which is dependent on calcium ions and calmodulin (3,4).

Relaxation of smooth muscle cells is achieved by increasing the cellular concentration of adenosine 3',5'-cyclic monophosphate (cAMP) or guanosine 3',5'-cyclic monophosphate (cGMP) (5). Accordingly, the addition of activators of adenylate cyclase to endothelial cell monolayers decrease the passage of macromolecules thus improving the barrier function of these monolayers (6,7). The increase of cAMP in endothelial cells is attended by a decrease in actin-myosin interaction, similar as seen in smooth muscle cell relaxation. Analogue to the contribution of cGMP to the relaxation of smooth muscle cells by nitric oxide, it may be hypothesized that cGMP is an important mediator of endothelial permeability. Few studies have been performed on cGMP regulating the permeability of endothelial cell monolayers, but their results are not unanimous (8-12). In the present study, we evaluated the role of cGMP in the permeability of human endothelial cell monolayers by the activation of soluble- and particulate guanylate cyclase as well as by the inhibition of nitric oxide synthase.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics from Corning (Corning, NY) or Costar (Cambridge, MA); and Transwells (diameter 0.65 cm; pore size 3 μm) from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al. (13). Human serum was obtained from the local blood bank and was prepared from fresh blood taken from healthy donors; this was pooled, and stored at 4°C. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY) and heat-inactivated before use (30 min, 56°C). Pyrogen-free human serum albumin (HSA) was purchased from CLB (Amsterdam, the Netherlands). Thrombin, horseradish peroxidase (HRP), sodium nitroprusside (SNP), 8-bromo-guanosine-3'-5'cyclic monophosphate (8-bromo-cGMP) and N^G-L-nitro-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Company (St. Louis, MO), forskolin

from Hoechst (La Jolla, CA) and isobutyl-methyl-xanthine (IBMX) from Janssen Chimica (Beerse, Belgium). Human 99-128 atrial natriuretic peptide (hANP) was purchased from Bissendorf Peptide GmbH (Wedermark, FRD).

Culture of endothelial cells

Human umbilical artery endothelial cells were isolated by the method of Jaffe et al. (14) and characterized previously (15). Isolation and characterisation of human endothelial cells from the pulmonary artery was performed as earlier described (16). Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin and penicillin/streptomycin. Cells were kept at 37°C under 5% CO_2/95% air. For passage studies, confluent monolayers of endothelial cells from umbilical artery (primary), pulmonary artery (first till third passage) or aorta (forth and fifth passage) were released with trypsin-EDTA (ethylene-diamine-tetraacetic acid) and seeded in high density on fibronectin-coated polycarbonate filters of the transwell system and cultured as described above (17,18). Medium was renewed every other day.

Extraction and assays of cyclic nucleotides

Cultured human endothelial cells were grown to confluence in 5 cm^2 wells. Medium of the monolayers was renewed with Medium 199 supplemented with 1% albumin one hour before the incubation period. Cells were preincubated with IBMX (1 mM) for 15 minutes. At the start of the experiment, stimulator agents were added to the medium and incubated for the indicated time interval. The medium was aspirated and frozen immediately at -70°C for later determination of cyclic nucleotides. Instantaneously upon removal of the medium, 3.5% perchloric acid (0.5 ml) as well as a small known amount of [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP were added to each well for the determination of the intracellular cyclic nucleotide concentration. Samples were transferred to Eppendorf reaction tubes and neutralized by potassium hydrogen carbonate (50% saturated). After centrifugation, the supernatants were collected and dried under a stream of nitrogen gas. The concentration of the intra- and extracellular cyclic nucleotides were determined using radio-immunoassays (Amersham, Amersham, UK) according to Steiner et al. (19) and corrected for the recoveries in the various samples.

Evaluation of the barrier function

Endothelial cells cultured on filters were used between 4 and 6 days after seeding. Exchange of macromolecules through the endothelial monolayers was investigated by assay of the transfer of horse radish peroxidase, which from the comparison with the passage of dextrans and proteins of various molecular weights had proven to be a useful marker protein (17,18). Passage of horseradish peroxidase through human endothelial cell monolayers was performed as described previously (17). Briefly, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) to form a tight monolayer. Before the experiment, cells were incubated for one hour in Medium 199 with 1% albumin. Monolayers were preincubated for 15 minutes with the various experimental agents in the upper and lower compartment. At the start of the experiment, 5 μg/ml horseradish peroxidase in Medium 199 with 1% albumin was added to the upper compartment of the Transwell-system in the presence or absence of thrombin (1 U/ml). Samples were taken from the lower compartment (at the other side of the endothelial monolayer) at various time intervals, and an equal amount of Medium 199-1% albumin was re-added to this lower compartment. Cells were kept at 37°C under 5% CO_2/95% air. All passage experiments were performed in triplicate. The peroxidase-concentration was determined in each sample as previously described (17).

The permeability coefficient (PC) was derived from Fick's law of diffusion and is determined by:

$$PC = \text{mass flux peroxidase}/([\text{peroxidase}]_{UC} - [\text{peroxidase}]_{LC})$$

where UC is the upper compartment and LC is the lower compartment. The mass flux of peroxidase is expressed in nanograms per square centimetre per second.

Because the initial passage of molecules proceeds linearly in time, the mass flux of peroxidase was calculated from the initial hours of passage, and the mean concentrations of the upper and lower compartments during this period were used to calculate the concentration difference. The PC was corrected for the contribution of the membrane as described by Siflinger-Birnboim et al. (20):

$$1/PC_{EC} = 1/PC_{EC\text{-}F} - 1/PC_{F}$$

where PC_{EC} represents the PC of the endothelial cell monolayer, PC_F the PC of the empty filter, and $PC_{EC\text{-}F}$ the PC determined for the filter and the endothelial cells together. The PC_F was determined at 37°C under identical conditions with separate fibronectin-coated filters that had been preincubated in culture medium for 24 hours.

Transendothelial electric resistance

The transendothelial electric resistance was measured as previously described (17). In short, an alternating current (50 μA) was passed across the monolayer (one pulse every minute). The measured electrical potential difference was used to calculate the electric resistance by Ohm's law and expressed in ohms times square centimeter.

Statistical analysis

Data are presented as mean $\pm$ SE unless stated otherwise. The effect of the various agents on the intracellular concentration of nucleotides and the effects on the passage rate of peroxidase were analyzed with the Mann-Whitney and Wilcoxon's rank sum test. Statistical significance was accepted for $p < 0.05$.

RESULTS

Effect of stimulation of guanylate- and adenylate cyclase on the intracellular concentrations of cGMP and cAMP

In human umbilical artery endothelial cells, the addition of hANP (10^{-7} M), an activator of particulate guanylate cyclase, rapidly increased the intracellular cGMP concentration (Fig. 1A). The increased generation of cGMP was sustained for at least four hours. Simultaneously, cGMP accumulated gradually in the extracellular compartment. Neither the intracellular cAMP concentration nor the extracellular accumulation of cAMP was affected by addition of hANP when compared to control experiments (Fig. 1B). During the incubation, cAMP slowly accumulated in the conditioning medium, in both hANP-treated and -untreated cells.

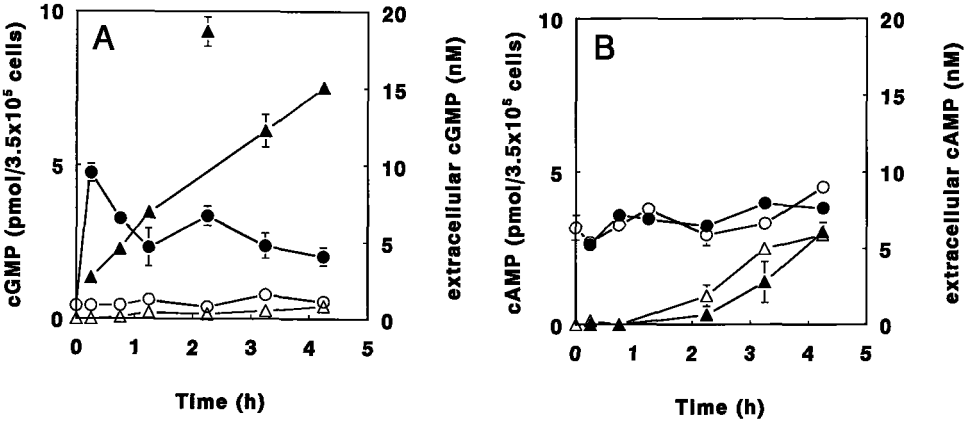

Figure 1: Effect of human atrial natriuretic peptide (hANP) on the intracellular (circles) and extracelluar (triangles) concentrations of cyclic nucleotides in human umbilical artery endothelial cells. Cells were incubated in Medium 199 with 1% albumin in the presence of 1 mM IBMX after addition of 10^{-7} M hANP (closed symbols) compared to control experiments (open symbols). A: Time course of the cGMP concentration. B: Time course of the cAMP concentration. Values represent the mean ± range of duplicate wells of a representative experiment.

Both hANP (10^{-7} M) as well as the activator of soluble guanylate cyclase SNP (10^{-4} M) evoked an average three-fold increase in the cGMP concentration in umbilical artery endothelial cells ($p < 0.05$, Table 1). In contrast to their effect on the cellular cGMP concentration, hANP and SNP did not affect the intracellular cAMP concentration. The adenylate cyclase activator forskolin (25 μM) increased the cAMP concentration three-fold without affecting the cGMP concentration ($p < 0.05$, Table 1). In human endothelial cells isolated from the pulmonary artery, an equivalent increase of the cellular cGMP concentration was demonstrated when the cells were incubated with hANP (10^{-7} M) or SNP (10^{-4} M). Accordingly, forskolin (25 μM) significantly increased the cAMP concentration threefold ($p < 0.05$). The addition of thrombin (1 U/ml) tended to increase the intracellular cGMP concentrations under basal conditions as well as after simultaneous stimulation with hANP and SNP in both endothelial cell types.

Table 1: Effect of activators of guanylate- and adenylate cyclase on the cellular content of cyclic nucleotides in human endothelial cells under basal conditions and after exposure to thrombin.

	Umbilical artery endothelial cells		Pulmonary artery endothelial cells	
Conditions	cGMP	cAMP	cGMP	cAMP
Control	1.1 ± 0.3 (7)	4.1 ± 0.7 (6)	0.7 ± 0.3 (3)	3.0 ± 1.0 (3)
+ hANP (10^{-7} M)	3.3 ± 0.3 (7)*	3.9 ± 0.8 (6)	3.1 ± 1.2 (3)	2.7 ± 1.0 (3)
+ SNP (10^{-4} M)	2.9 ± 0.9 (7)*	3.7 ± 0.8 (6)	1.3 ± 0.4 (3)	2.2 ± 0.7 (3)
+ forskolin (25 μM)	1.0 ± 0.3 (6)	12.2 ± 2.9 (6)*	0.8 ± 0.4 (2)	10.8 ± 0.8 (2)
+ thrombin (1 U/ml)	1.9 ± 0.3 (5)	4.9 ± 1.2 (5)	1.5 ± 0.5 (2)	2.3 ± 0.1 (2)
+ thrombin + hANP (10^{-7} M)	4.0 ± 1.3 (5)*	6.0 ± 1.2 (5)	3.5 ± 1.7 (2)	2.4 ± 0.2 (2)
+ thrombin + SNP (10^{-4} M)	5.8 ± 3.0 (5)*	4.7 ± 1.3 (5)	1.4 ± 0.6 (2)	2.8 ± 0.1 (2)

Values are expressed as pmol/3.5 x 10^5 cells and represent the mean ± SE of independent experiments performed in duplicate (number within paracentheses). Endothelial cell cultures were obtained from different donors. Cells were incubated in Medium 199 + 1% human serum albumin (control) and preincubated for 15 min. in the presence of 1 mM IBMX. Cellular cyclic nucleotides were assayed 15 min. after addition of the indicated factors. * Indicates statistical difference from control value ($p < 0.05$, paired data). hANP: human atrial natriuretic peptide, SNP: sodium nitroprusside.

Effect of cGMP- and cAMP-increasing agents on the passage of macromolecules through monolayers of human endothelial cells

When the permeability of human umbilical artery endothelial cells was evaluated in medium 199 containing 1% albumin, the passage rate of horse radish peroxidase (HRP) was 6.6 ± 1.2 ng HRP/h/cm^2 (mean ± SE, 13 experiments). This corresponds to a permeability coefficient of 3 x 10^{-7} cm/sec. When the cellular cAMP concentration was increased upon the addition of forskolin (25 μM), the permeability coefficient declined to 6 x 10^{-8} cm/sec ($p < 0.05$). Addition of thrombin (1 U/ml) increased the HRP passage rate an average five-fold ($p < 0.05$, Table 2). Preincubation with forskolin inhibited the thrombin-increased passage rate (circa 85% reduction) to a level similar as the passage rate in cells not treated with thrombin ($p < 0.05$). In contrast to the cAMP-elevating agents, the cGMP-elevating agents hANP (10^{-7} M) and SNP (10^{-4} M) as well as 8-bromo-cGMP (1 mM) only slightly decreased the passage rate of peroxidase under basal conditions. However, when these cells were incubated with thrombin the addition of 8-bromo-cGMP or SNP but not hANP elicits a significant 25% inhibition of the passage rate ($p < 0.05$).

In endothelial cell monolayers of the human pulmonary artery, the mean basal passage rate of peroxidase was three-fold higher (permeability coefficient 9×10^{-7}). Thrombin increased the passage rate of peroxidase seven-fold on the average in these cells ($p < 0.05$, Table 2). This increased permeability was significantly reduced by (pre)incubation of the endothelial cells with hANP (10^{-7} M), SNP (10^{-4} M) or 8-bromo-cGMP (1 mM) ($p < 0.05$). Figure 3 shows that hANP reduced the thrombin-enhanced permeability in a concentration dependent way. Under basal conditions none of the cGMP-enhancing agents reduced the endothelial permeability to a significant level (Table 2). On the other hand, elevation of the cellular cAMP concentration by the adenylate cyclase-stimulating agent forskolin was highly effective in reducing both the basal and thrombin-enhanced permeability of human endothelial cell monolayers.

Table 2: Effect of activators of guanylate- and adenylate cyclase on the passage of peroxidase through monolayers of human endothelial cells under basal conditions and after exposure to thrombin.

	peroxidase passage (ng/h/cm^2)	
Conditions	Umbilical artery endothelial cells	Pulmonary artery endothelial cells
Control	6.6 ± 1.2 (13)	16.4 ± 3.3 (5)
+ hANP (10^{-7} M)	5.7 ± 1.5 (9)	14.7 ± 3.6 (5)
+ SNP (10^{-4} M)	5.4 ± 1.2 (7)	13.8 ± 3.6 (5)
+ 8-bromo-cGMP (1 mM)	6.3 ± 1.2 (7)	13.8 ± 4.8 (5)
+ Forskolin (25 μM)	1.8 ± 0.6 (4)*	8.1 ± 1.2 (5)*
+ Thrombin (1 U/ml)	35 ± 6 (13)*	118 ± 65 (5)*
+ Thrombin + hANP (10^{-7} M)	31 ± 11 (9)	53 ± 30 (5) ¶
+ Thrombin + SNP (10^{-4} M)	23 ± 8 (7) ¶	60 ± 32 (5) ¶
+ Thrombin + 8-bromo-cGMP (1 mM)	27 ± 4 (7) ¶	34 ± 13 (5) ¶
+ Thrombin + Forskolin (25 μM)	5 ± 2 (4) ¶	9 ± 1 (5) ¶

Values represent the mean ± SE of independent experiments performed in triplicate (number within parentheses). Endothelial cell cultures were obtained from different donors. The passage rate of peroxidase (5 μg/ml in upper compartment) was determined in Medium 199 + 1% human serum albumin (control) between the first and second hour of incubation. The basal passage rate of peroxidase corresponds to a permeability coefficient of 3.6×10^{-7} and 9.5×10^{-7} cm/sec for the two cell types. * Indicates statistical difference from control value ($p < 0.05$, paired data). ¶ Indicates statistical difference from the addition of thrombin only ($p < 0.05$, paired data).

Effect of cGMP- and cAMP-increasing agents on the transendothelial electrical resistance across human endothelial cell monolayers

The transendothelial electrical resistance (TEER) of the human umbilical endothelial cell monolayers used in these experiments varied between 17 and 21 Ohm.cm^2. The TEER increased significantly after addition of forskolin under basal conditions ($p < 0.05$, Fig. 4). After the addition of thrombin, the TEER sharply decreased whereas preincubation with 8-bromo-cGMP or forskolin significantly prevented the fall of electrical resistance ($p < 0.05$) indicating that the tightness of the intercellular junctions was enhanced. In endothelial cell monolayers of the pulmonary artery, the TEER varied between 11 and 19 Ohm.cm^2 (four experiments). Similar as seen with monolayers of umbilical cells, the TEER decreased upon addition with thrombin. The decrease in electrical resistance was slightly inhibited by 8-bromo-cGMP and forskolin, but this did not reach statistical significance (data not shown).

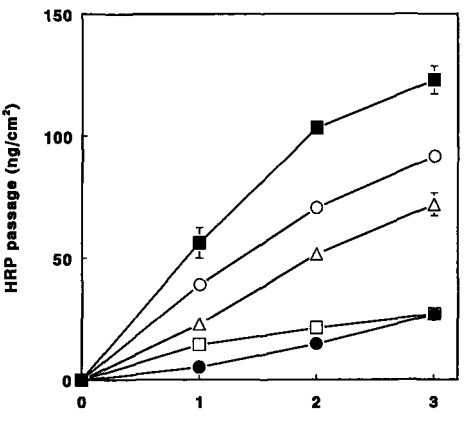

Figure 2: Effect of thrombin on the passage of horseradish peroxidase (HRP) through human pulmonary artery endothelial monolayers compared to control experiments (closed circles). Cells were incubated in Medium 199 with 1% albumin in the presence of 1 U/mL thrombin only (closed squares) or simultaneously with 1 U/ml thrombin and 10^{-7} M hANP (open triangles), 10^{-4} M sodium nitro-prusside (SNP, open circles) or 25 μM forskolin (open squares). hANP, SNP and forskolin were added to the cells 15 minutes prior to the addition of thrombin. Values are the mean $\pm$ SE of triplicate filters of a representative experiment.

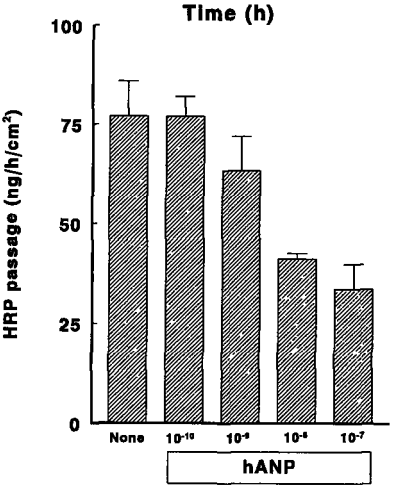

Figure 3: Effect of increasing concentrations of hANP (10^{-10} - 10^{-7} M) on the passage rate of HRP through monolayers of human pulmonary artery endothelial cells after exposure to thrombin. Cells were incubated in Medium 199 with 1% albumin in the presence of 1 U/mL thrombin. Passage rate (ng/h/cm^2) was determined between the first and second hour of incubation. Values represent the mean $\pm$ SE of triplicate filters of a representative experiment.

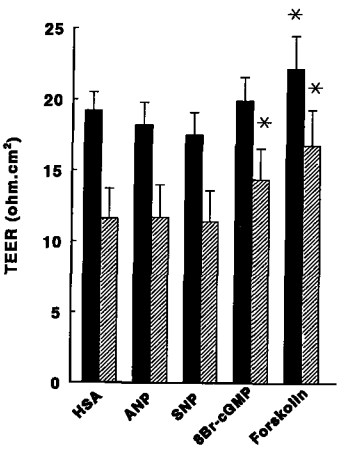

Figure 4: Transendothelial electrical resistance (TEER) in monolayers of human umbilical artery endothelial cell monolayers obtained from different donors. Cells were preincubated for 15 min. in medium 199 with 1% albumin and in the presence of 10^{-7} M ANP, 10^{-4} M SNP, 1 mM 8-bromo-cGMP or 25 μM forskolin (solid bars) and incubated for another 15 min after the addition of 1 U/mL thrombin (hatched bars). Values represent the mean $\pm$ SE of six independent experiments performed in duplicate. * Statistical significant compared to control values ($p < 0.05$).

Effect of inhibition of nitric oxide on the permeability of human endothelial monolayers

To evaluate if nitric oxide formation was involved in the regulation of endothelial permeability, nitric oxide synthase was inhibited by the addition of the compitative inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). In comparison with the incubation with thrombin only (15 minutes), the simultaneous incubation of thrombin and L-NAME (10^{-4} M) suppressed the cGMP concentration from 2.9 $\pm$ 0.4 to 1.9 $\pm$ 0.2 pmol/3.5 x 10^5 cells ($n = 6$, $p < 0.05$; control values 1.8 $\pm$ 0.1 pmol/3.5 x 10^8 cells). Concurrently the permeability of tight umbilical artery endothelial monolayers increased from 45 $\pm$ 6 to 75 $\pm$ 13 ng HRP/h/cm^2 ($n = 14$, $p < 0.05$). Because the endogenous formation of nitric oxide and subsequent elevation of the cellular cGMP level may affect the effectivity of exogenously added stimulators of cGMP formation, we have studied the effects of hANP and 8-bromo-cGMP on the permeability of human endothelial monolayers, which were (pre)incubated with L-NAME. In the majority of endothelial cell cultures studied, the addition of L-NAME (10^{-4} M) increased the passage of peroxidase through the endothelial monolayers, the extent of which depended on the cell strain. This was found under basal conditions and, more pronounced, after stimulation of the cells by thrombin. Three representative experiments are shown in Table 3, two cultures (experiments 1 and 3) which significantly responded to L-NAME, and one (experiment 2) in which the permeability did not change after addition of L-NAME. In experiments 1 and 3, the addition of hANP and 8-bromo-cGMP to umbilical or pulmonary artery endothelial cells caused an obvious decrease in the passage rate of peroxidase and (partially) compensated the increase induced by L-NAME (Table 3). The time course of these effects are shown in Fig. 5. The addition of 8-bromo-cGMP inhibited the additional increase in permeability mediated by L-NAME under basal conditions (Fig. 5, inset) as well as after exposure to thrombin (Fig. 5).

Table 3: Effect of L-NAME on the thrombin induced passage of peroxidase through monolayers of human endothelial cells.

Conditions	Peroxidase passage (ng/2h/cm²)	
	Thrombin	Thrombin + L-NAME
Experiment 1		
Umbilical artery endothelial cells	83 ± 13	202 ± 9
+ hANP (10^{-7} M)	62 ± 6	119 ± 7
+ 8-bromo-cGMP (1 mM)	40 ± 7	67 ± 11
Experiment 2		
Umbilical artery endothelial cells	45 ± 5	48 ± 6
+ 8-bromo-cGMP (1 mM)	32 ± 2	27 ± 6
Experiment 3		
Pulmonary artery endothelial cells	48 ± 7	67 ± 16
+ hANP (10^{-7} M)	25 ± 6	44 ± 5
+ 8-bromo-cGMP (1 mM)	19 ± 2	20 ± 3

Values represent the means ± SE of three independent representive experiments performed in triplicate. Endothelial cell cultures were obtained from different donors. The passage of peroxidase (5 μg/ml in upper compartment) was determined in Medium 199 + 1% albumin and thrombin (1 U/ml), two hours after the addition of the indicated factors. When indicated, cells were preincubated with N^G-L-nitro-arginine methyl ester (L-NAME 10^{-4} M) for one hour.

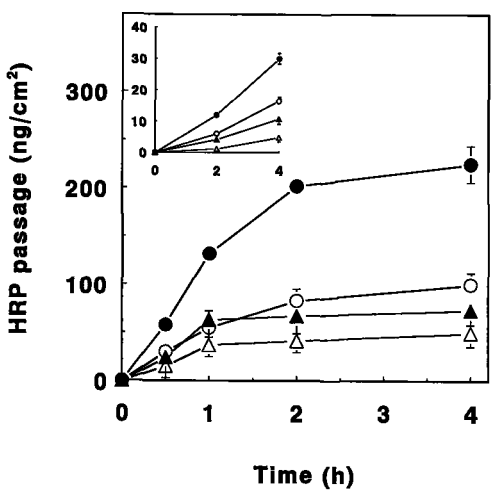

Figure 5. Effect of nitric oxide on the passage rate of horseradish peroxidase through human umbilical endothelial monolayers under basal conditions (inset) and after exposure to thrombin. Cells were incubated in Medium 199 with 1% albumin with (closed symbols) and without (open symbols) 10^{-4} M N^G-L-nitro-arginine methyl ester (l-NAME) only or simultaneously with 1 mM 8-bromo-cGMP (triangles). Values are the mean ± SE of triplicate filters of a representive experiment.

DISCUSSION

In this study, selective activators of guanylate cyclase and adenylate cyclase were used to assess the role of cGMP in the regulation of human endothelial permeability. Several lines of evidence indicate that stimulation of cGMP production can improve the barrier function of human endothelial cells under our experimental conditions. The addition of 8-bromo-cGMP reduced the thrombin-enhanced passage of the marker protein peroxidase through endothelial monolayers, in agreement with previous studies on bovine endothelial cells (8,10,21). Additionally, both ANP which activates particulate guanylate cyclase via specific B-receptors (22) and SNP which activates soluble guanylate cyclase (23) induced parallel changes in the passage rate of peroxidase. Furthermore, inhibition of the endogenous production of nitric oxide by L-NAME (24) enhanced in many endothelial cell cultures the passage for macromolecules. As nitric oxide also activates soluble guanylate cyclase in endothelial cells it is most likely that L-NAME acted on endothelial permeability by decreasing the generation of cGMP.

hANP and SNP induced similar increases in the cellular cGMP concentrations of human umbilical- and pulmonary artery endothelial cells. This finding is in agreement with previous reports on human umbilical vein and bovine aorta endothelial cells (25-27). Although SNP was effective in reducing the permeability of thrombin-stimulated umbilical and pulmonary artery endothelial cells, ANP decreased the passage of peroxidase much more effectively in endothelial cell monolayers from the pulmonary artery than in those from the umbilical artery. This contrasts with the decrease in permeability upon addition of the cAMP-elevating agent forskolin which was equally effective in all cell strains. These data suggest that the two endothelial cell types respond differently to ANP. Recently, the existence of C-type ANP receptors has been demonstrated on blood platelets in addition to the B-type ANP receptors (28). These C-type ANP receptors, which upon ligand binding activates a G_i-protein reducing the cellular cAMP level, could have resulted in an increase of endothelial permeability. Such a mechanism may counteract the reduction in permeability by ANP evoked via its B-receptor. However, we could not demonstrate a decrease in the cellular cAMP concentration after exposure of our cells to ANP, in accordance with a report of Kent et al. (29) on bovine endothelial cells.

Upon the addition of 8-bromo-cGMP or forskolin, an activator of adenylate cyclase, the TEER increased, suggesting that the tightness of the intercellular junctions was enhanced. These alterations of the TEER were in agreement with - albeit less pronounced than - the observed changes in permeability. The inability of hANP and SNP to reduce the thrombin-induced increase in TEER was unexpected, as significant effects of these mediators were

demonstrated on the endothelial permeability. Because changes in TEER reflect the passage of ions, it is possible that the changes in the passage of macromolecules and ions do not proceed completely synchronously.

It is likely that the suppressive effects of cAMP and cGMP on endothelial permeability, which is influenced by actin-myosin interaction (2,4), are comparable with their inhibitory effects on smooth muscle cell contraction. The mechanism of vascular smooth muscle relaxation by cGMP is still not completely understood. It has been demonstrated that cGMP-dependent protein kinase mediates smooth muscle cell relaxation (23), probably causing a reduction of intracellular calcium (30). A contribution of the cGMP-dependent protein kinase in endothelial cell relaxation has been disputed, as cGMP-dependent protein kinase activity was hardly detectable in cultured bovine endothelial cells (31). However, unpublished data from our laboratory on human endothelial cells indicate that elevation of cGMP in endothelial cells indeed can interfere with the accumulation of cytoplasmic calcium ions (Draijer, Atsma and van Hinsbergh, manuscript in preparation). This contrasts with previous findings in bovine endothelial cells in which a dissociation between the effect of cGMP on permeability and the cellular calcium concentration was found (21). This finding suggests that cGMP may exert effects independently of influencing intracellular calcium.

cGMP may influence the cellular cAMP concentration by competition for phosphodiesterase(s) and/or by directly activating cGMP-activated cAMP-phosphodiesterase or inhibiting cGMP-inhibited cAMP-phosphodiesterase (32). Selective inhibition of cGMP-inhibited cAMP-phosphodiesterase has been shown to attenuate pulmonary vasoreactivity in isolated rat lungs (33). Therefore it remains to be investigated if differences in the cellular content of cGMP-dependent cAMP-phosphodiesterases, may also contribute to the differential endothelial responses to cGMP. Expression of different types or quantities of cGMP-dependent phosphodiesterases may contribute to the results obtained with pig aorta endothelial cells (9) and in one report on bovine pulmonary endotheial cells (12) on the one hand, in which cGMP induced an increase in permeability; and data on bovine and human endothelial cells on the other hand, in which cGMP reduces the thrombin-induced permeability (8,10,21,32).

Stimulation of endothelial cells by thrombin causes a rapid increase in the cytoplasmatic concentration of calcium ions (34,35). The then formed calcium/calmodulin complex not only stimulates the contractive interaction of actin and myosin (4), but can also activate the constitutive form of nitric oxide synthase in endothelial cells (36). The formed nitric oxide increases the cellular cGMP concentration by activation of soluble guanylate cyclase. Therefore, we have considered if autocrine nitric oxide production may mask the effects of cGMP-generating agents and hence may account for the different cGMP responses in the various endothelial cell cultures that we have investigated. After inhibition of nitric oxide

synthesis, we found indeed an inhibition of the thrombin-induced increase in cGMP and an enhancement of permeability, suggesting that nitric oxide may act as an endogenous modulator of endothelial permeability. Whether this effect is limited to endothelial cells of large vessels, which are known to contain considerable amounts of nitric oxide synthase (37,38), or also involves specific types of microvascular endothelial cells has still to be established. Furthermore, it should be noted that these *in vitro* data on the effects of nitric oxide on endothelial permeability can not be directly extrapolated to the *in vivo* situation. The effect of nitric oxide on the vascular smooth muscle tonus may affect the capillary perfusion pressure and consequently extravasation of colloids. Studies on the effect of L-NAME on vascular permeability *in vivo* are not unamious. L-NAME is found to decrease the adenosine and bradykinine induced permeability in the hamster cheek pouch (39) whereas L-NAME increased the permeability in the coronary circulation of the rat (40).

The different endothelial responses to the cGMP-generating hormone ANP suggest that ANP may have distinct effects in various vascular beds. *In vivo*, ANP has opposite effects on the permeation of water and macromolecules in the pulmonary and systemic circulation; in rats the administration of ANP elicited an extravasation of albumin and plasma water in the systemic circulation whereas the vascular permeation in the pulmonary circulation was reduced (41). Analogously, it has been demonstrated that ANP attenuates the formation of edema in the isolated, perfused animal lung after administration of several toxic agents (42-44), whereas the administration of ANP augments capillary filtration in the human fore-arm (45). In this way the hormone may counteract central volume overload, while it increases the barrier function of the pulmonary endothelium to prevent pulmonary edema.

In conclusion, the presented data indicate that - in addition to cAMP - cGMP is an important mediator of endothelial permeability in umbilical and pulmonary artery endothelial cells. As endogenous nitric oxide generation also enhances the endothelial cGMP concentration, nitric oxide may act as an endogenous permeability modulator in arterial endothelial cells (46).

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CHAPTER 4

CYCLIC GMP AND NITRIC OXIDE MODULATE THROMBIN-INDUCED ENDOTHELIAL PERMEABILITY.

REGULATION VIA DIFFERENT PATHWAYS IN HUMAN AORTA AND UMBILICAL VEIN ENDOTHELIAL CELLS.

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ABSTRACT

Previous studies have demonstrated that guanosine-3',5'-cyclic monophosphate (cGMP) and adenosine-3',5'-cyclic monophosphate (cAMP) reduce the endothelial permeability for fluids and macromolecules when the endothelial permeability is increased by thrombin. In this study we have investigated the mechanism by which cGMP improves the endothelial barrier function and examined whether nitric oxide (NO) can serve as an endogenous modulator of endothelial barrier function. Thrombin increased the passage of macromolecules through human umbilical vein and human aorta endothelial cell monolayers and concomitantly increased the cytoplasmic calcium ion concentration *in vitro*. Inhibition of these increases by the intracellular calcium chelator BAPTA indicated that cytoplasmic calcium ion elevation contributes to the thrombin-induced increase in endothelial permeability. The cGMP-dependent protein kinase activators 8-Bromo-cGMP (8-Br-cGMP) and 8-(4-Chlorophenylthio)-cGMP (8-PCPT-cGMP) decreased thrombin-induced passage of macromolecules. Two pathways accounted for this observation. Activation of cGMP-dependent protein kinase by 8-PCPT-cGMP decreased the accumulation of cytoplasmic calcium ions in aorta endothelial cells, and hence reduced the thrombin-induced increase in permeability. On the other hand, in umbilical vein endothelial cells, cGMP-inhibited-phosphodiesterase (PDE III) activity was mainly responsible for the cGMP-dependent reduction of endothelial permeability. The PDE III-inhibitors Indolidan (LY195115) and SKF94120 decreased the thrombin-induced increase in permeability by 50% in these cells. Thrombin treatment increased cGMP formation in the majority, but not all cell cultures. Inhibition of NO production by N^G-L-nitro-arginine methyl ester (L-NAME) enhanced the thrombin-induced increase in permeability, which was restricted to those cell cultures which displayed an increased cGMP formation after addition of thrombin. Simultaneous elevation of the endothelial cGMP concentration by atrial natriuretic peptide, sodium nitroprusside or 8-Br-cGMP prevented the additional increase in permeability induced by L-NAME.

These data indicate that cGMP reduces thrombin-induced endothelial permeability by inhibition of the thrombin-induced calcium accumulation and/or by inhibition of cAMP degradation by PDE III. The relative contribution of these mechanisms differs in aorta and umbilical vein endothelial cells. NO can act *in vitro* as an endogenous permeability-counteracting agent by raising cGMP in endothelial cells of large vessels.

INTRODUCTION

The endothelium, the inner lining of blood vessels, regulates the extravasation of fluid and macromolecules. Impairment of the barrier function of the endothelium results in vascular leakage and edema. This can occur by exposure to toxic agents, after stimulation of the endothelium by vasoactive substances, or during inflammation, in particular in postcapillary venules. It is generally believed that the increase in endothelial permeability induced by vasoactive substances is caused by contraction of endothelial cells.[1-7] Endothelial contraction involves interaction of actin and non-muscle myosin, which is activated by a calcium/calmodulin (Ca^{2+}/CaM) and ATP-dependent phosphorylation of the myosin light chain (MLC) by MLC-kinase.[5,8,9] The barrier function of endothelial cells is improved both *in vivo* and *in vitro* by agents that increase the intracellular cAMP concentration.[10-15] An increase in cellular cAMP was found to be accompanied by a reduced degree of phosphorylation of the myosin light chain in cultured endothelial cells.[9] Several *in vitro* studies have shown that elevation of the cGMP concentration also reduces endothelial permeability in large vessel endothelial cells.[16-18] The modulating effect of cGMP is most prominent when the endothelial permeability has been increased, for instance by thrombin or oxidants, whereas it is minor or absent under basal conditions.[16,18,19] In perfused rat lungs, stimulation of cGMP production by atrial natriuretic peptide also reduced oxidant-induced vascular leakage.[20] However, the mechanism by which cGMP reduces oxidant- and thrombin-enhanced permeability is not known.

The process of endothelial cell contraction resembles the regulation of actin-myosin interaction in smooth muscle cells and platelets. The effects of cGMP on smooth muscle relaxation are thought to be mediated via cGMP-dependent protein kinase, which affects the intracellular calcium metabolism.[21-23] In smooth muscle and several other cell types cGMP also contributes indirectly by inhibiting phosphodiesterase type III (PDE III), which results in a decreased breakdown of cAMP.[24,25] In the present study we have investigated, in human umbilical vein and aorta endothelial cells, whether cGMP regulates endothelial permeability by affecting the regulation of the cytoplasmic calcium ion accumulation or by inhibiting PDE III activity.

Stimulation of the influx of calcium ions in endothelial cells not only causes endothelial cell contraction, but also results in the release of several endothelial products, including prostacyclin and nitric oxide (NO). Production of NO is due to the calcium/calmodulin-dependent activation of the constitutive nitric oxide synthase, which is predominantly present in muscular vessel endothelial cells.[26,27] The production of NO not only reduces the contraction of smooth muscle cells and counteracts platelet activation, but it also stimulates

guanylate cyclase in the endothelial cell itself. Because the cGMP thereby generated may counteract the stimulus-induced increase in permeability, we wondered whether the production of NO attenuates the contraction of endothelial cells. Our data points to a possible counter-regulatory role of nitric oxide on the regulation of endothelial permeability.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics from Corning (Corning, NY, USA) or Costar (Cambridge, MA, USA); and Transwells (diameter 0.65 cm, pore size 3 μm) from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al.[28] Human serum was obtained from the local blood bank and was prepared from fresh blood taken from healthy donors; the sera were pooled, and stored at 4°C. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY, USA) and heat-inactivated before use (30 min, 56°C). Pyrogen-free human serum albumin (HSA) was purchased from the Central Laboratory of Blood Transfusion Service (Amsterdam, the Netherlands). Horseradish peroxidase EC 1.11.1.7 type I (HRP), sodium nitroprusside(SNP), 8-bromo-guanosine-3'-5'cyclicmonophosphate(8-Br-cGMP), N^G-L-nitro-arginine methyl ester (L-NAME) and fluorescein isothiocyanate-dextrans (dextran-FITC) with a molecular masses of 35 600, 38 900 and 487 000 D were obtained from Sigma Chemical Company (St. Louis, MO, USA); bovine α-thrombin from LEO Pharmaceutical Products (Ballerup, Denmark); forskolin from Hoechst (La Jolla, CA, USA); isobutyl-methyl-xanthine (IBMX) from Janssen Chimica (Beerse, Belgium); SKF96365 from Biomol Research Laboratories (Plymouth Meeting, PA, USA); BAPTA-AM and fura 2-AM from Molecular Probes (Eugene, OR, USA); 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-PCPT-cGMP) from Biolog Life Science Institute (Bremen, Germany); ionomycin from Calbiochem Corporation (La Jolla, CA, USA); [^{14}C]-sucrose from Dupont NEN (Bad Hamburg, Germany); and human atrial natriuretic factor-(99-128) was purchased from Bissendorf Peptide GmbH (Wedermark, Germany). SKF94120 was a gift from Smith Kline & French Laboratories Ltd. (Welwyn Garden, England); Rolipram (ZK62711), a gift from Schering Aktiengesellschaft (Berlin, Germany); and Indolidan (LY195115), a gift from Lilly Research Laboratories (Indianapolis, IN, USA).

Isolation and culture of endothelial cells

Human umbilical vein endothelial cells were isolated by the method of Jaffe et al.[29] and characterized as described previously.[30] Isolation and characterization of human endothelial cells from the pulmonary artery and aorta were performed as earlier described.[31] The blood vessels of human origin were obtained according to the guidelines of the Institutional Review Board of the University Hospital Leiden. Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were kept at 37°C under 5% CO_2/95% air. For the evaluation of the barrier function, confluent monolayers of endothelial cells from umbilical vein (primary), pulmonary artery (first, second or third passage) or aorta (fourth and fifth passage) were released with trypsin-EDTA and seeded in high density on fibronectin-coated polycarbonate filters of

the Transwell™ system and cultured as described by Langeler et al.[32,33] Medium was renewed every other day.

Evaluation of the barrier function

Endothelial cells cultured on filters were used between 4 and 6 days after seeding. Exchange of macromolecules through the endothelial monolayers was investigated by assay of the transfer of horseradish peroxidase (HRP) and dextran-FITC. Passage of HRP through human endothelial cell monolayers was performed as described previously.[32] Briefly, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) to form a tight monolayer. Before the start of the experiment, cells were incubated for one hour in Medium 199 with 1% albumin. In pretreatment, the cells were incubated for 15 minutes with 8-Br-cGMP (1-1000 μM), 8-PCPT-cGMP (1-1000 μM), SNP (0.1 mM), ANP (10^{-7} M), SKF94120 (100 μM), Indolidan (100 μM) or Rolipram (100 μM) in the upper and lower compartment. BAPTA/AM (10 μM) and L-NAME (100 μM) were preincubated for one hour to achieve sufficient loading. At the start of the experiment, 5 μg/ml horseradish peroxidase in Medium199 with 1% albumin was added to the upper compartment of the Transwell™ system in the presence or absence of thrombin (1 U/ml). Samples were taken from the lower compartment (at the other side of the endothelial monolayer) at various time intervals, and an equal volume of Medium 199 containing 1% albumin was re-added to this lower compartment. Cells were kept at 37°C under 5% CO_2/95% air. All passage experiments were performed in triplicate. The concentration of HRP was derived from the HRP activity in each sample with peroxide and tetramethyl benzidine (TMB), as substrate and expressed as ng passed per cm^2 in a certain time interval. The permeability coefficient (PC) was derived from Fick's law of diffusion and is determined by: $PC = \text{mass flux peroxidase} / ([\text{peroxidase}]_{UC}-[\text{peroxidase}]_{LC})$
where UC is the upper compartment and LC is the lower compartment. The mass flux of HRP is expressed in $ng.cm^{-2}.h^{-1}$. Because the initial passage of molecules proceeds linearly in time, the mass flux of peroxidase was calculated from the initial hour of passage, and the mean concentrations of the upper and lower compartments during this period were used to calculate the concentration difference. The PC was corrected for the contribution of the filter membrane (less than 0.5%):

$$1/PC_{EC} = 1/PC_{EC\text{-}F} - 1/PC_F$$

where PC_{EC} represents the PC of the endothelial cell monolayer, PC_F the PC of the empty filter, and $PC_{EC\text{-}F}$ the PC determined for the filter and the endothelial cells together. The PC_F was determined at 37°C under identical conditions with separate fibronectin-coated filters that had been preincubated in culture medium for 24 hours. The passage of fluorescein-isothiocyanate-labeled dextran (FITC-dextran; input upper compartment 1 mg/ml) was determined similarly with the use of an inverted fluorescence microscope equipped with a photometer and a scanning stage, and operated by a microprocessor.[34]

Extraction and assays of cyclic nucleotides

Cultured human endothelial cells were grown to confluence in 5 cm^2 wells. Medium of the monolayers was renewed with Medium 199 supplemented with 1% albumin, with or without 100 μM L-NAME, one hour before the incubation period. Cells were preincubated for 15 min with IBMX (1 mM) to prevent degradation of cyclic nucleotides by phosphodiesterases. At the start of the experiment, thrombin was added to the medium and incubated for 15 min. Immediately upon removal of the medium, 3.5% perchloric acid (0.5 ml) as well as a small known amount of [^{3}H]-cyclic GMP or [^{3}H]-cyclic AMP were added to each well for the determination of the intracellular cyclic nucleotide concentration. The cell lysates were transferred to Eppendorf reaction tubes and neutralized by using potassium hydrogen carbonate (50% saturated). After centrifugation, the supernatants were

collected and dried under a stream of nitrogen gas. The concentration of the intracellular cyclic nucleotides was determined using radio-immunoassays (Amersham, Amersham, UK), according to Steiner et al.[35] and corrected for the recoveries in the various samples.

Measurement of the intracellular calcium ion concentration

Endothelial cells were cultured on 5 cm^2 glass coverslips and loaded with fura 2 by incubation with 2 μM fura 2/AM for 45 to 60 min at 37°C in M199 supplemented with 1%HSA. Then, the cells were washed three times with Tyrode buffer. The coverslips were mounted in a teflon two compartment incubation dish, incubated in 1 ml Tyrode buffer, and placed in a temperature controlled micro-incubator.[36,37] The two compartment dish allows the exposure of the two halves of the same culture to different treatment. In this way, the effect of thrombin on the intracellular calcium ion concentration in one half can be compared to the effect of thrombin, in the presence of 8-PCPT-cGMP, 8-Br-cGMP, BAPTA or SKF96365 in the other half of the same culture. fura 2 fluorescence was measured with an imaging dual-wavelength fluorescence microscope, which consisted of an inverted microscope body (Leitz Diavert, Wetzlar, Germany) equipped with a 20x fluorite objective (Nikon, Badhoevedorp, The Netherlands) and a mercury light source (HBO-100, Osram, Montgomery, NY, USA). A filterwheel (Sutter, Novato, CA, USA) allowed the selection of excitation filters of 340 nm and 380 nm. Emission fluorescence was led through a 490 nm high-pass filter and imaged by a high-sensitivity SIT camera (Hamamatsu C2400-08, Herrsching, Germany). The resulting video signal was digitized by a frame-grabber board (PCVISIONplus™, Imaging Technologies, Woburn, MA, USA) in a PC-AT 486 computer. Spatial resolution of the images was 256*256 pixels, with an eight bits intensity resolution. Every 3.6 s a pair of images at 340 nm and 380 nm excitation wavelength was made. Off-line, background fluorescence was substracted and the 340 nm image was divided by the 380 nm image on a pixel-by-pixel basis, yielding a ratio image. Statistical analysis was performed using dedicated image processing software (TIM, Difa, Breda, The Netherlands). The mean intracellular calcium ion concentration ($[Ca^{2+}]_i$) was determined from a field of fifty cells and was calculated by the equation:

$$[Ca^{2+}]_i = K_d * \beta * [(R-R_{min})/(R_{max}-R)] \quad \text{in nM}$$

in which R represents the ratio of the fluorescence values at 340 nm and 380 nm; R_{max} and R_{min} are the maximal and minimal ratio values, respectively, being determined after each experiment by addition of 1 μM ionomycin and 10 mM EGTA, respectively; β represents the ratio of the fluorescence at 380 nm of free fura 2 and fura 2 completely saturated with calcium (3.6); the K_d, the dissociation constant of the fura 2-Ca^{2+} complex, was assumed to be 224 nM at 37°C, according to Grynkiewicz et al.[38]

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis as indicated in the text was performed with the Mann-Whitney and Wilcoxon's rank sum test. Statistical significance was assumed if $p < 0.05$.

RESULTS

Elevation of cytoplasmic calcium ion concentration during thrombin-induced increase in endothelial permeability

FITC dextrans and HRP, which has a Stokes radius similar to that of albumin, were used as marker molecules to assay the permeability of human endothelial cell monolayers for macromolecules. Upon addition of 1 U/ml thrombin the permeability of human umbilical vein endothelial cell monolayers for ^{14}C-sucrose (360 D), 38,900 D FITC-dextran, HRP and 487,000 D FITC-dextran increased two-, five-, seven- and fifteenfold, respectively (average values of ten different cultures; not shown). The increase in permeability was detectable rapidly after addition of thrombin (Figure 1A) and lasted for at least one hour. It was accompanied by an immediate decrease in the trans-endothelial electrical resistance (40-60% reduction; not shown). Thrombin also rapidly enhanced the passage rate of HRP fivefold through monolayers of human aorta endothelial cells (Figure 1D).

Thrombin induced an immediate rise in $[Ca^{2+}]_i$ in both endothelial cell types (Figure 1B, 1E). This increase was abolished by the intracellular calcium ion chelator BAPTA. The elevation of the $[Ca^{2+}]_i$ was caused by a rapid release of calcium ions from intracellular stores and an influx of extracellular calcium ions (Figure 1B), since $[Ca^{2+}]_i$ accumulation was reduced by the calcium entry blocker SKF96365[39] and by incubation in calcium-free medium supplemented with EGTA (not shown). Further evidence that elevation of the $[Ca^{2+}]_i$ is also important for the prolonged thrombin-mediated increase in endothelial permeability was obtained using the intracellular calcium chelator BAPTA. BAPTA reduced the thrombin-mediated increase in permeability in a concentration-dependent way (Figure 1C, 1F). In the presence of 10 μM BAPTA the thrombin-induced increase in permeability was reduced to 50 $\pm$ 9% in umbilical vein endothelial cells (5 independent cultures, $p < 0.05$) and to 53 $\pm$ 20% in aorta endothelial cells (3 independent experiments with cells from two different donors).

cGMP induces a simultaneous reduction of thrombin-enhanced permeability and rise of $[Ca^{2+}]_i$ in aorta endothelial cells

The thrombin-enhanced permeability was reduced in human umbilical vein and aorta endothelial cell monolayers by the cell membrane-permeant cGMP analogue 8-Br-cGMP (Table 1). Under basal conditions 8-Br-cGMP was less or not effective on endothelial permeability. When another cGMP-analogue 8-PCPT-cGMP was used, the thrombin-increased permeability was reduced in aorta endothelial cell monolayers to 50 $\pm$ 3 and 33 $\pm$ 8% in the presence of 0.1 mM and 1 mM 8-PCPT-cGMP, respectively (4 experiments),

but was not affected in umbilical endothelial cell monolayers (with 1mM 8-PCPT-cGMP 101 ± 14% of thrombin stimulated counterparts; 7 experiments).

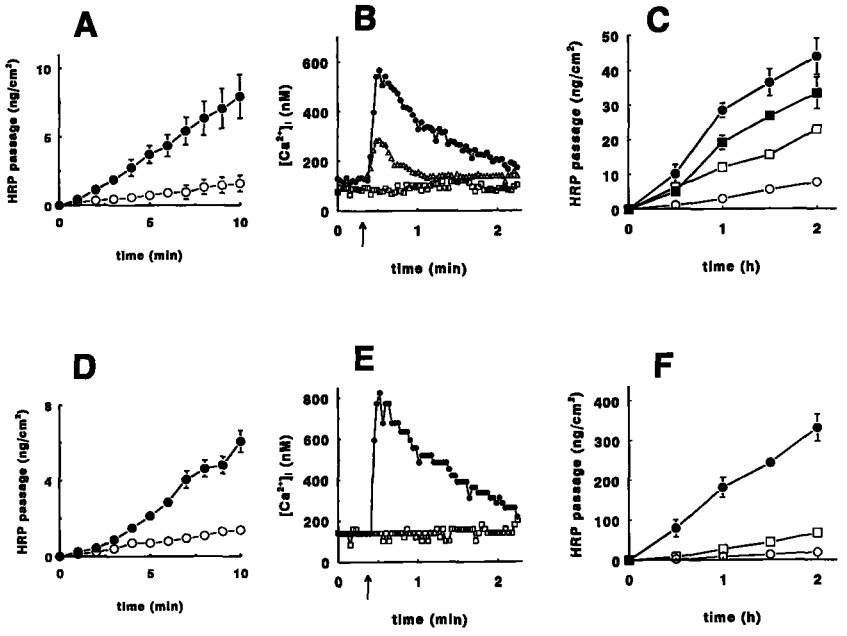


Figure 1: Graphs showing effect of thrombin on the $[Ca^{2+}]_i$ and permeability of human umbilical vein (A through C) and human aorta (D through F) endothelial cell monolayers. A and D, Early time courses show the passage of horseradish peroxidase (HRP) under basal conditions (○) and after stimulation with 1 U/ml thrombin (●), which is added at t=0 (mean ± SEM of six determinations). The thrombin-induced permeability is significantly different from the basal permeability after 3 min ($p < 0.05$). Passage of HRP was determined as described in "Materials and Methods". B and E, The increase in $[Ca^{2+}]_i$ after addition, indicated by an arrow, of 1 U/ml thrombin (●), was prevented by addition of the intracellular Ca^{2+} chelator BAPTA-AM (10 μM, □). In the presence of the Ca^{2+} entry blocker SKF96365 (100 μM, △) $[Ca^{2+}]_i$ was markedly reduced. Each graph represents the mean of three representative recordings with different batches of umbilical vein endothelial cells and one representative recording with aorta endothelial cells. Similar results were obtained with 25 μM EGTA in calcium-free buffer instead of SKF96365 (not shown). C and F, Time courses show the passage of HRP in hours under basal conditions (○) and after stimulation with 1 U/ml thrombin (●). The thrombin-induced passage of HRP through endothelial cell monolayers was partly prevented in monolayers, that were preincubated for 1 hour with BAPTA-AM (1 μM, ■ and 10 μM, □) (mean ± SEM of triplicate cultures).

Table 1. Effect of 8-Bromo-cGMP (1 mM) on the passage of horseradish peroxidase (HRP) through human umbilical vein and human aorta endothelial cell monolayers under basal conditions and after stimulation with 1 U/ml thrombin.

Endothelial cells	**Addition**	**HRP Passage ($ng.cm^{-2}.h^{-1}$)**	
		Control	8-Br-cGMP
Umbilical vein EC	None	10 ± 2 (22)	7 ± 2 (15)*
	Thrombin	71 ± 14 (22)#	48 ± 7 (22)*
Aorta EC	None	43 ± 14 (7)	31 ± 12 (7)
	Thrombin	193 ± 57 (7)#	90 ± 29 (7)*

Endothelial permeability was determined in medium199 supplemented with 1% human serum albumin and with or without 1mM 8-Br-cGMP. HRP passage was determined after an one hour time interval as described in the Methods section. Data represent the mean ± SEM of the number of experiments given in parentheses. * 8-Br-cGMP-treated cells statistically different from their control counterparts ($p < 0.05$); # thrombin-stimulated vs. control cells statistically different (umbilical vein $p < 0.001$, aorta $p < 0.05$).

The cGMP-analogues activate cGMP-dependent protein kinase with a similar potency, but have relatively little effect on cAMP-dependent protein kinase.[40,41] In addition, 8-PCPT-cGMP acts selectively on cGMP-dependent protein kinase as compared to cGMP-regulated phosphodiesterases, whereas 8-Br-cGMP is less specific in this respect.[40] Both 8-PCPT-cGMP and 8-Br-cGMP decreased the thrombin-enhanced permeability for macromolecules at low concentrations (1-30 μM) in aorta endothelial cells (Figure 2). This suggests that activation of cGMP-dependent protein kinase is indeed involved.

Determination of the $[Ca^{2+}]_i$ in fura-2-loaded endothelial cells revealed that the thrombin-induced elevation of $[Ca^{2+}]_i$ was markedly reduced by a preincubation with 8-PCPT-cGMP in aorta endothelial cells. On the other hand, the $[Ca^{2+}]_i$ rise was only marginally attenuated by 8-PCPT-cGMP in umbilical vein endothelial cells (Table 2; Figure 3). The cAMP-analogue 8-Br-cAMP (0.1-1 mM) did not change the thrombin-induced elevation of the $[Ca^{2+}]_i$ in either cell type (not shown).

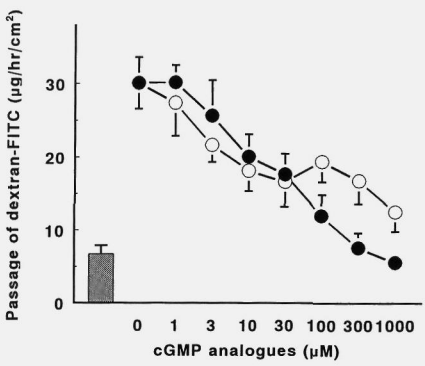


Figure 2. Graph showing the passage of a FITC-labeled dextran (38 900 D) through human aorta endothelial cell monolayers after stimulation with 1 U/ml thrombin, measured in the presence of different concentrations of 8-Bromo-cGMP (○) or 8-PCPT-cGMP (●). The basal passage (bar) increased fourfold after thrombin stimulation. The elevated passage was concentration-dependently reduced by the cGMP analogues. The passage of HRP was reduced similarly (not shown). Data are means of two different cultures, performed in duplicate.

Table 2: Reduction of thrombin-induced rise in the $[Ca^{2+}]_i$ in endothelial cells by 8-PCPT-cGMP.

	$[Ca^{2+}]_i$ (nM)		
Endothelial cells	basal	thrombin	thrombin + 8-PCPT-cGMP
Umbilical vein EC (n=10)			
peak value	120 ± 6	899 ± 85	726 ± 85
after 1 min		388 ± 57	269 ± 22
Aorta EC (n=7)			
peak value	111 ± 5	761 ± 188	228 ± 29*
after 1 min		486 ± 102	167 ± 20*

The intracellular calcium concentration was determined as described in the Methods section. The peak value and 1 min after stimulation with 1 U/ml thrombin of the intracellular calcium ion concentration (in nM) is presented with and without a fifteen minutes preincubation of 100 μM 8-PCPT-cGMP in aorta and umbilical vein endothelial cells. Data are mean ± SEM of the number of determinations given in parenthesis. * 8-PCPT-cGMP-treated cells are statistically different from their control counterparts ($p < 0.05$).

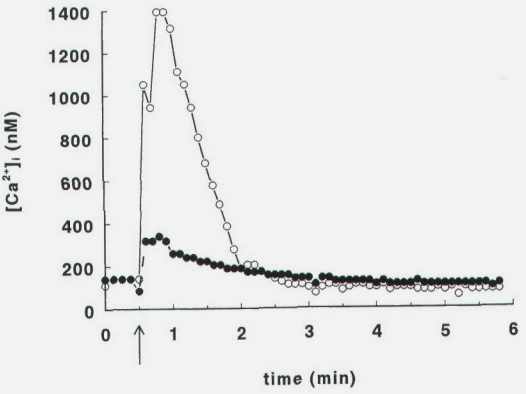
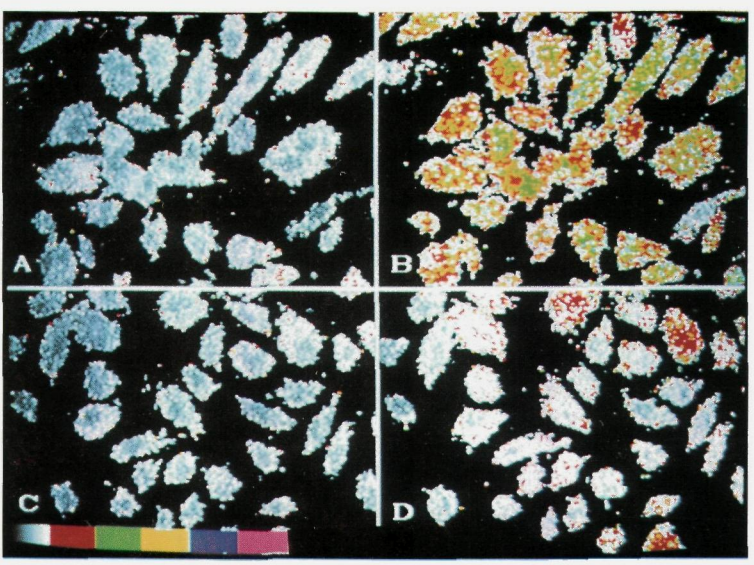


Figure 3. Effect of 8-PCPT-cGMP on the $[Ca^{2+}]_i$ in thrombin-stimulated human aorta endothelial cells.
Top, Graph showing that preincubation of endothelial cells with 100 μM 8-PCPT-cGMP (●) reduced the increase in $[Ca^{2+}]_i$ induced by 1 U/ml thrombin (○). The arrow indicates the time point of thrombin addition. Bottom, Video microscope image of a part of the culture before (A and C) and 10 seconds after stimulation with thrombin (B and D). The intensity of the fluorescence ratio 340/380 nm, which is represented in pixels, is reduced by 15 min preincubation of the cells with 8-PCPT-cGMP (C and D). The fluorescence intensity bar represents, from left to right, an increase in $[Ca^{2+}]_i$.



cGMP-inhibited cAMP phosphodiesterase activity in umbilical vein endothelial cells

The discrepancy between the effects of 8-PCPT-cGMP and 8-Br-cGMP on thrombin-enhanced permeability of human umbilical vein endothelial cells, suggests the existence of an additional regulatory target, by which cGMP may affect permeability. Therefore, we investigated if a cGMP-inhibited cAMP phosphodiesterase activity (PDE III) contributed, additionally, to the reducing effect of cGMP on the passage of macromolecules through thrombin-stimulated endothelial cell monolayers. SKF94120 and Indolidan, two specific inhibitors of the phosphodiesterase type III (cGMP-inhibited cAMP PDE) were used. When cAMP levels were measured after thrombin-stimulation in umbilical vein endothelial cells, cAMP increased from 1.2 $\pm$ 0.2 pmol in the absence of PDE III-inhibitors to 1.9 $\pm$ 0.4 pmol/3.5 x 10^5 cells in the presence of thrombin and SKF94120 ($p < 0.05$) and tended to increase to 1.9 $\pm$ 0.5 pmol in the presence of thrombin and Indolidan (eight different cell cultures). In the absence of thrombin both inhibitors slightly increased cAMP approximately 30% compared to control. SKF94120 and Indolidan, as well as Rolipram, an inhibitor of the PDE type IV (cAMP specific phosphodiesterase), inhibited the thrombin-induced increase of the passage of macromolecules through umbilical vein endothelial cell monolayers (Figure 4). SKF94120 slightly reduced the thrombin-induced HRP passage in aorta endothelial cells to 89 $\pm$ 7% (four cultures). The basal permeability was not changed by SKF94120 in either cell type.

Inhibition of nitric oxide synthesis by L-NAME intensifies the thrombin-induced elevation of endothelial permeability

The rise in $[Ca^{2+}]_i$ after addition of thrombin stimulates the constitutive calcium/calmodulin-dependent nitric oxide synthase. Nitric oxide (NO) activates guanylate cyclase, which leads to cGMP generation. In agreement with observations by other authors,[42] we found that thrombin augmented the intracellular cGMP concentration in tight endothelial cell monolayers of umbilical vein from 1.3 $\pm$ 0.2 to 2.4 $\pm$ 0.4 pmol/3.5 x 10^5 cells ($p < 0.01$; 11 different cultures, assayed after 15 min in the presence of IBMX). This increase in cGMP concentration is apparently due to NO generation, because (pre)incubation of the cells with the competitive NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) prevented the thrombin-induced increase in these cells (1.4 $\pm$ 0.2 pmol cGMP/3.5 x 10^5 cells; $p < 0.05$ as compared to thrombin-stimulated cells). cGMP was not significantly altered when these cells were (pre)incubated with L-NAME alone (1.6 $\pm$ 0.2 pmol cGMP/3.5 x 10^5 cells). Therefore, we wondered whether the thrombin-induced increase in permeability may be partly attenuated/counteracted by generation of NO. If so, addition of L-NAME would be expected to increase thrombin-induced permeability. In 37 different cultures of human endothelial cell monolayers the thrombin-enhanced permeability increased

by 51 ± 13% after preincubation of the cells for one hour with 100 μM L-NAME ($p < 0.005$). Although this effect is highly significant, considerable variation was observed between cultures.

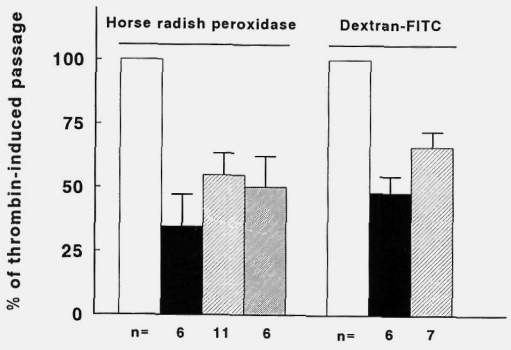


Figure 4. Bar graph showing the effect of phosphodiesterase inhibitors on the passage of HRP and FITC-dextran (35 600 Da) through monolayers of umbilical vein endothelial cells. The endothelial cells were stimulated with 1 U/ml thrombin in the presence of Rolipram (100 μM, filled bars), SKF94120 (100 μM, hatched bars) or Indolidan (100 μM, narrowly hatched bar), or without addition of PDEi (open bars). Permeability values after stimulation with thrombin (= 100%) were 76 ± 19 $ng.cm^{-2}.h^{-1}$ for HRP and 7.6 ± 0.7 $\mu g.cm^{-2}.h^{-1}$ for dextran-FITC. All conditions reduced the passage of both tracer molecules ($p < 0.05$ for Rolipram and Indolidan, $P < 0.01$ for SKF94120). Data are mean ± SEM of the indicated number of different cultures.

In Figure 5 the effect of L-NAME on the thrombin-enhanced permeability is plotted as a function of the thrombin-enhanced permeability. The effect of L-NAME was significant in 23 cultures, which had a thrombin-enhanced permeability for HRP that was less than 100 $ng.cm^{-2}.h^{-1}$ (42 ± 4 $ng.cm^{-2}.h^{-1}$ *vs.* 69 ± 9 $ng.cm^{-2}.h^{-1}$). This effect could not be demonstrated in the cultures, which displayed a relatively high permeability after thrombin-stimulation (14 cultures with a mean permeability of 217 ± 21 $ng.cm^{-2}.h^{-1}$). In the latter cultures, the thrombin-induced increase in permeability could still be reduced by an elevation of the intracellular cGMP content by 8-Br-cGMP or ANP (not shown). In the responsive cultures, L-NAME enhanced the thrombin-induced increase in permeability in a concentration-dependent manner (Figure 6). Furthermore, the additional increase caused by L-NAME was completely prevented by agents that raise cGMP: atrial natriuretic peptide (ANP), sodium nitroprusside (SNP) and 8-Br-cGMP (Figure 7). This was also observed in human pulmonary artery endothelial cells (Figure 7b). Additionally, a significant increase of the passage of HRP through umbilical vein endothelial cell monolayers after thrombin stimulation by L-NAME from 100 (thrombin) to 130 ± 18% (thrombin with L-NAME) was decreased by SNP to 105 ± 12% (thrombin with L-NAME and SNP; 7 cultures of different donors). L-NAME was ineffective on the basal permeability regardless of the basal passage rate. To evaluate whether the observed lack of response to L-NAME was associated with an

impaired formation of NO and/or cGMP, cGMP and thrombin-enhanced permeability were determined in 10 independent cultures of umbilical vein endothelial cells. The cGMP concentration was increased after thrombin stimulation from 0.8 ± 0.1 to 2.5 ± 0.4 pmol/3.5 x 10^5 cells in cultures with a low thrombin-induced permeability ($p < 0.05$, five different cultures), but remained unchanged in cultures with an high permeability (0.6 ± 0.1 *vs.* 0.9 ± 0.4 pmol/3.5x10^5 cells, respectively, five different cultures).

To obtain further mechanistic information, cyclic nucleotides and cytoplasmic calcium concentrations were assayed after addition of thrombin and L-NAME in aorta and umbilical vein endothelial cells, in the absence of IBMX. A transient (50-100%) increase in cGMP was observed, which peaked at 5-6 min after addition of thrombin. In aorta endothelial cells, the cGMP concentration increased from 0.39 ± 0.09 to 0.62 ± 0.06 pmol cGMP/3.5x10^5 cells 5 min after thrombin addition. Preincubation of the cells with L-NAME reduced the cellular cGMP concentration to 0.24 ± 0.13 pmol in those cells. In the same aorta endothelial cell culture, the peak value of $[Ca^{2+}]_i$ after stimulation by thrombin (606 ± 170 nM) was additionally increased by preincubation of the cells with L-NAME to 1015 ± 184 nM ($p < 0.05$; 24 determinations). These observations are consistent with the suggestion that NO mediated cGMP generation partially reduces the accumulation of $[Ca^{2+}]_i$ after stimulation of aorta endothelial cells by thrombin.

In umbilical vein endothelial cells no change in $[Ca^{2+}]_i$ was observed. On the other hand, in the absence of IBMX, cAMP increased after stimulation with thrombin from 1.9 ± 0.5 to 2.6 ± 0.2 pmol/3.5 x 10^5 cells (3 different cultures). The thrombin-induced increase in cAMP was reduced by L-NAME to 2.1 ± 0.1 pmol/3.5 x 10^5 cells.

DISCUSSION

In this study we have presented evidence that endogenous production of nitric oxide attenuates the thrombin-induced increase in permeability by a cGMP-dependent mechanism. Elevation of the cGMP concentration acts on the regulation of permeability by suppressing the elevation of cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$) via cGMP-dependent kinase and by elevation of the cellular cAMP concentration via the cGMP-dependent inhibition of PDE III. The relative contribution of these mechanisms is different in human aorta and umbilical vein endothelial cells.

Figure 5. Graph showing the effect of L-NAME on thrombin-stimulated endothelial permeability in cultures of three endothelial cell types. The ratio of the passage of HRP after 1 U/ml thrombin stimulation with and without the nitric oxide synthase inhibitor L-NAME (100 μM) is plotted against the HRP passage in $ng.cm^{-2}.h^{-1}$ after thrombin stimulation without L-NAME. No additional effect of L-NAME upon the thrombin-induced passage is marked with a horizontal line at a ratio of one. The passage is shown 1 hour after thrombin-stimulation for umbilical vein (○), aorta (■) and pulmonary artery (●) endothelial cell monolayers. The response to L-NAME is inversely correlated to the permeability of HRP in the presence of thrombin. Each point represents the mean of a triplicate determination.

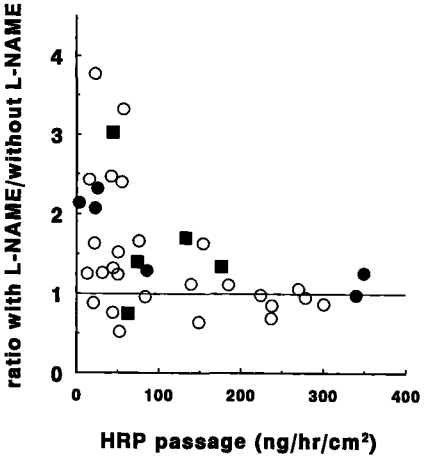
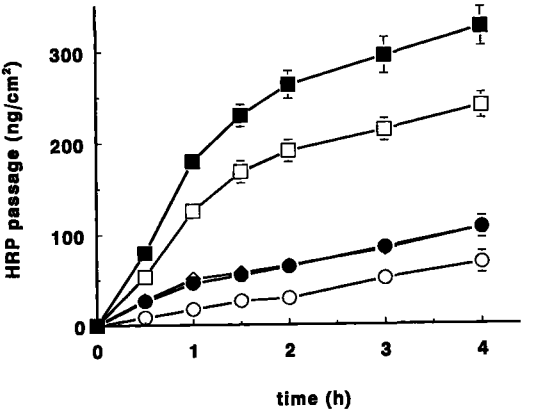


Figure 6. Graph showing the effect of L-NAME on the thrombin-induced increase in endothelial permeability. The time courses of the HRP passage through human umbilical vein endothelial cell monolayers are presented, under basal condition (medium 199 supplemented with 1% human serum albumin) or after stimulation with 1 U/ml thrombin, in the presence or absence of L-NAME. The basal HRP passage (○) is increased upon addition of thrombin (●) and is further elevated in combination with L-NAME (1 μM ◇; 10 μM □; and 100 μM ■). Data are mean ± SEM of triplicate filters.



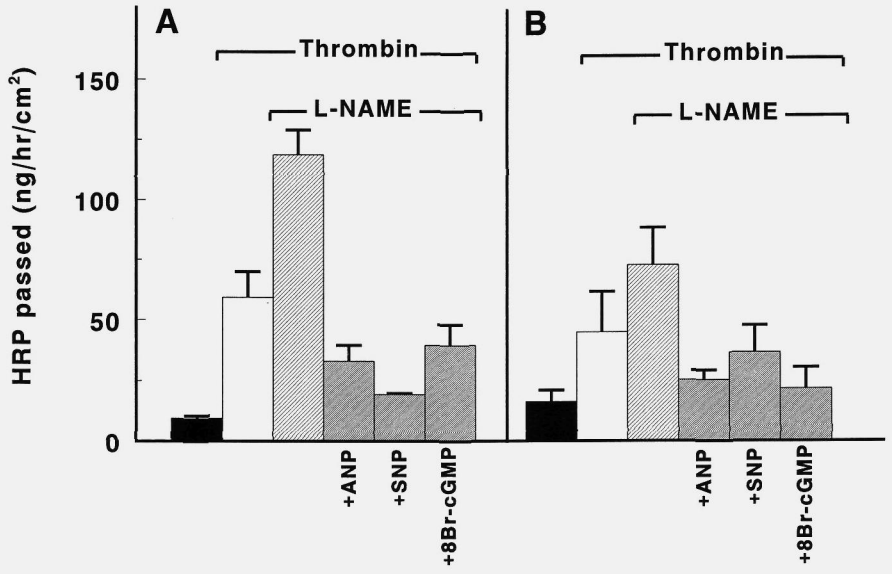


Figure 7: Bar graphs showing that cGMP-elevating agents reduce the L-NAME-dependent enhancement of thrombin-stimulated permeability of human aorta and pulmonary artery endothelial cell monolayers. L-NAME (100 μM, hatched bars) enhances the increase in permeability induced by 1 U/ml thrombin (open bars); the permeability under control conditions is indicated by filled bars. Simultaneous addition of the cGMP-elevating agents atrial natriuretic peptide (ANP, 10^{-7} M), sodium nitroprusside (SNP, 10^{-4} M) and 8-Br-cGMP (1 mM) reduced the increased permeability induced by thrombin and L-NAME. A, human aorta endothelial cell monolayers, two different cultures; B, human pulmonary artery endothelial cell monolayers, three different cultures. Data are mean $\pm$ SEM.

Mechanisms involved in cGMP-dependent reduction of thrombin-stimulated endothelial permeability

Previous reports have shown that elevation of the cellular cGMP concentration reduces the increase in endothelial permeability induced by thrombin.[16,19] Our data confirms these observations and identifies two mechanisms by which cGMP acts on endothelial permeability: reduction of the increase in $[Ca^{2+}]_i$ induced by thrombin, and elevation of the cellular cAMP concentration by inhibition of PDE III. Calcium ions are involved in the induction of endothelial contraction.[4,8,17] Our data with the intracellular Ca^{2+} ion chelator BAPTA demonstrates a direct relation between the rise in $[Ca^{2+}]_i$ and a rapid and prolonged increase in endothelial permeability after exposure to thrombin. The sustained elevation of the permeability, after $[Ca^{2+}]_i$ has returned to basal level, suggests the onset of other intracellular events.[6] Two lines of evidence indicate that cGMP interferes with the calcium-dependent

increase in permeability, in particular in human aorta endothelial cells. First, the increase in permeability induced by thrombin was reduced by 8-PCPT-cGMP and 8-Br-cGMP at concentrations, at which they selectively activate cGMP-dependent protein kinase, as compared to the activation of the cAMP-dependent protein kinase.[40,41] We recently found that endothelial cells from human aorta, but not of umbilical vein contain a considerable amount of cGMP-dependent protein kinase (Draijer et al., manuscript in preparation). Second, direct assay of the $[Ca^{2+}]_i$ in fura 2-loaded endothelial cells demonstrated a reduced accumulation of Ca^{2+} ions in the presence of 8-PCPT-cGMP. Reduction of cytoplasmic Ca^{2+} accumulation by activation of cGMP-dependent protein kinase is expected to reduce the Ca^{2+}/calmodulin-dependent phosphorylation of the myosin light chain kinase and the subsequent actin-non muscle myosin interaction.[5,8,9] It is unlikely that cGMP reduces the Ca^{2+} response via interaction with the regulatory subunit of the cAMP-dependent protein kinase,[43] because 1 mM 8-Br-cAMP did not influence the thrombin-stimulated Ca^{2+} response. This observation is in accordance with the inability of cAMP-elevating agents to reduce accumulation of cytoplasmic Ca^{2+} induced by histamine.[44] The mechanism by which cGMP affects the accumulation of Ca^{2+} ions in aorta endothelial cells is not known. On the analogy of smooth muscle cells one may expect that the cyclic nucleotide can induce a decrease in Ca^{2+} influx or an increase in calcium efflux.[45,46] Ca^{2+} efflux from vascular smooth muscle cells was found to be stimulated by cGMP via Na^+/Ca^{2+}-exchange.[47] Alternatively, it has been suggested that cGMP-dependent protein kinase activity causes reduction of cytoplasmic Ca^{2+} via suppression of inositol-1,4,5-trisfosfate formation[48] or via the stimulation of Ca^{2+}-ATPase pumps.[22,23] Further studies are needed to elucidate whether one or several of these mechanisms are involved in the cGMP-dependent reduction of $[Ca^{2+}]_i$ in endothelial cells.

In addition to reducing cytoplasmic Ca^{2+} accumulation, cGMP also affects endothelial cell permeability by inhibiting cGMP-inhibited cAMP-phosphodiesterase (PDE III). PDE III has been demonstrated previously in endothelial cells[49,50] and has been implicated in the control of endothelial permeability.[49] Inhibition of PDE III lowers the cellular breakdown of cAMP and enhances the steady state level of cAMP.

Many studies have demonstrated that elevation of the cAMP concentration in endothelial cells can reduce endothelial permeability *in vivo*[10,11,51] and *in vitro*.[14,15] cAMP activates the cAMP-dependent protein kinase, which interferes with endothelial contraction by several mechanisms including reduction of the phosphorylation of the myosin light chain.[9] Involvement of PDE III in cGMP-dependent reduction of the increased permeability mediated by thrombin was demonstrated in our study using two specific PDE III inhibitors, Indolidan and SKF94120.[52,53] PDE III inhibition was found in human umbilical vein endothelial cells in particular, whereas only a small effect of the PDE III inhibitors was observed in human aorta endothelial cells. Thus, the PDE III activity may be different in various endothelial cell

types. Alternatively, the PDE III activity of endothelial cells from aorta and umbilical vein may have been altered to a different degree during subculturing of the cells. In umbilical vein endothelial cells 8-PCPT-cGMP did not decrease the thrombin-induced permeability and in parallel reduced the thrombin-stimulated $[Ca^{2+}]_i$ rise only slightly. The fact that 8-Br-cGMP reduced the permeability of these cells can be explained by an inhibitory action of 8-Br-cGMP on PDE III, a property which is less prominent for 8-PCPT-cGMP.[40,41]

NO acts as an endogenous modulator of endothelial cell function

The notion that cGMP can modulate endothelial $[Ca^{2+}]_i$ puts forward the question whether nitric oxide (NO), which induces cGMP generation by activation of soluble guanylate cyclase not only in smooth muscle cells and platelets[54,55] but also in endothelial cells,[56,57] acts as an endogenous counter-regulatory molecule. Under normal non-inflammatory conditions, NO is generated in endothelial cells by the constitutive NO synthase, the activity of which depends amongst others on Ca^{2+}/calmodulin.[58,59] Thrombin evokes a rapid increase in $[Ca^{2+}]_i$ in endothelial cells. In accordance with the aforementioned feature of the constitutive NO synthase, thrombin causes a rapid and sustained elevation of NO generation[60] and an increase of the cGMP level (Reference 42 and the present study) in human endothelial cells. Inhibition of NO synthase by L-NAME[61,62] prevented cGMP accumulation. The enhancement of the thrombin-induced increase of endothelial permeability caused by preincubation of the cells with L-NAME suggests that the NO/cGMP generation indeed modulates endothelial contraction, at least partly by attenuating the cytoplasmic Ca^{2+} accumulation. This suggestion is further strengthened by the observation that the L-NAME-induced increase in permeability was abolished by adding agents that increase the cellular cGMP production independently of NO synthase. Furthermore, a preincubation with L-NAME caused an additional increase of the thrombin-induced $[Ca^{2+}]_i$ accumulation. Shin et al.[56] obtained comparable results with bovine aorta endothelial cells, in which ATP-induced $[Ca^{2+}]_i$ accumulation was enhanced by the NO-synthesis inhibitor L-NMMA. Thrombin-induced NO/cGMP formation may, in umbilical vein endothelial cells, increase intracellular cAMP via inhibition of cAMP degradation. This was suggested by cAMP accumulation after thrombin-stimulation in the presence of the PDE III-inhibitors SKF94120 and Indolidan. Additionally, thrombin-induced cAMP accumulation was blocked by L-NAME. A counter-regulatory role of NO/cGMP is probably to be found not only in the regulation of endothelial permeability, but also in other Ca^{2+}-dependent processes in the endothelial cells such as the generation of prostacyclin,[63] PAF[64] and NO itself,[59] and the release of von Willebrand factor and tissue-type plasminogen activator.[65] Indeed, Buga et al.[66] reported recently, that NO is able to modulate its own generation.

Our observation that those endothelial cell monolayers, that displayed a rather high permeability after exposure to thrombin (permeability coefficient > 5.5 x 10^{-6} cm/s), were not affected by L-NAME was surprising, but not contrary to our previous findings. These cells, for unknown reasons, are probably defective in the generation of NO and/or cGMP. This suggestion is favoured by the observations that thrombin did not enhance the cellular cGMP concentration in such cells and that the thrombin-induced increase in permeability is excessively high. It further strengthens the hypothesis that Ca^{2+}-regulated NO production prevents excessive contraction of endothelial cells and impairment of their barrier function.

In conclusion, cGMP elevation attenuates the thrombin-induced increase in permeability of endothelial monolayers *in vitro*. cGMP can act via two pathways: cGMP reduces elevation of thrombin-stimulated $[Ca^{2+}]_i$ and reduces cAMP-degradation by inhibition of the PDE III activity. We postulate that autocrine nitric oxide can act as a permeability-counter-regulatory agent in endothelial cells.

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CHAPTER 5

EXPRESSION OF CGMP-DEPENDENT PROTEIN KINASE I AND PHOSPHORYLATION OF ITS SUBSTRATE VASP IN HUMAN ENDOTHELIAL CELLS OF DIFFERENT ORIGIN

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ABSTRACT

Previous studies demonstrated that the thrombin-induced permeability of endothelial cell monolayers is reduced by elevation of cyclic GMP. Now, the presence of cGMP-dependent protein kinase (cGMP-PK) immunoreactivity and activity in various types of human endothelial cells (EC), and its role in the reduction of thrombin-induced endothelial permeability was investigated. cGMP-PK type I was demonstrated in freshly isolated EC from human aorta and iliac artery as well as in cultured EC from human aorta, iliac vein and foreskin microvessels. Addition of the selective cGMP-PK activator 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP) to these EC caused phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), an established cGMP-PK substrate, which is localized at cell-cell contact sites of confluent EC. cGMP-PK I expression decreased during serial passage of EC, which correlated with a diminished ability of 8-pCPT-cGMP to induce VASP phosphorylation. Preincubation of aorta and microvascular endothelial cell monolayers with 8-pCPT-cGMP caused a 50% reduction of the thrombin-stimulated permeability, as determined by measuring the peroxidase passage through endothelial cell monolayers on porous filters. Furthermore, the thrombin-induced rise in cytoplasmic calcium ion concentration was strongly attenuated by the cGMP-PK activator in fura-2 loaded aorta EC. In contrast, cGMP-PK could not be demonstrated in freshly isolated and cultured human umbilical vein EC. Incubation of umbilical vein EC with 8-pCPT-cGMP did not cause VASP phosphorylation, and had no effect on the thrombin-induced increases in cytoplasmic calcium and endothelial permeability. These data indicate that cGMP-PK I is expressed in various types of human macro- and microvascular EC, but is absent or expressed in very low amounts in umbilical vein EC. cGMP-PK I expression in EC may be important in the regulation of endothelial permeability and the release of factors involved in vasoregulation and hemostasis.

INTRODUCTION

The endothelium, which lines the blood vessels, actively regulates the extravasation of fluid, nutrients and hormones into the tissues. In vascular leakage, intercellular exchange of macromolecules and fluid over this barrier is enhanced by endothelial cell (EC) contraction.[1,2,3,4] This process can be mimicked in vitro by using tight monolayers of human EC on porous filters.[5,6] We and other investigators have demonstrated that elevation of cGMP in EC counteracts the thrombin- and oxidant-induced increase in endothelial permeability in vitro.[7,8,9,10] Intracellularly, cGMP acts via cGMP receptor proteins, which can be divided into distinct classes such as cGMP-regulated ion channels, cGMP-regulated phosphodiesterases, cGMP-dependent protein kinases (cGMP-PK), and perhaps even cAMP-dependent protein kinases.[11,12,13] cGMP-PK I phosphorylates certain cellular proteins including vasodilator-stimulated phosphoprotein (VASP), a recently cloned proline-rich protein which is associated with actin filaments and focal contacts.[14,15] Furthermore, cGMP-PK plays an important role in the signal transduction pathway by which endothelium-derived nitric oxide (NO) and other cGMP generating agents induce smooth muscle cell relaxation and inhibition of platelet activation.[12,16] Activation of cGMP-PK I by cGMP indirectly inhibits agonist-induced activation of phospholipase C and the accumulation of cytoplasmic Ca^{2+} in platelets,[17,18] and causes a reduction of the agonist-induced accumulation of cytoplasmic Ca^{2+} in smooth muscle cells.[19,20] This reduces cell activation and contraction. Although it may be assumed that a similar mechanism underlies the cGMP-dependent reduction of endothelial contraction and vascular leakage, it has been difficult to detect cGMP-PK I in vascular EC by immunofluorescence studies using intact tissues.[11,12,21,22] On the other hand, our previous study[23] suggested that the modulating effect of cGMP on agonist-induced endothelial permeability was mediated by cGMP-PK in human aorta EC in vitro, while another mechanism, probably via phosphodiesterase III, contributed to the cGMP-induced reduction of permeability of umbilical vein endothelial cell monolayers.[23] Another isoform of cGMP-PK, type II, which is implicated in the regulation of salt and fluid secretion in the intestine, was recently shown also to be present in tissues outside the intestine.[24] Therefore, we now investigated the presence and possible activation of cGMP-PK I and II in cultures and freshly obtained isolations of various human endothelial cell types. Our data demonstrate expression and activation of cGMP-PK I in human aorta and foreskin microvascular EC, but not in umbilical vein EC. This different expression of cGMP-PK I closely agrees with differences in the effects of cGMP on the cytoplasmic Ca^{2+} concentration and the regulation of permeability in these cells, and points to a autoregulatory role of NO-derived cGMP in EC.

Preliminary results of this work has been presented in abstract form.[25,26]

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mM *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics from Corning (Corning, NY, USA) or Costar (Cambridge, MA, USA); and Transwells (diameter 0.65 cm, pore size 3 μm) from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al.[27] Human serum obtained from a local blood bank was prepared from freshly taken blood of healthy donors. Human sera were pooled and stored at 4°C. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY, USA) and heat-inactivated before use (30 min, 56°C). Pyrogen-free human serum albumin (albumin) was purchased from the Central Laboratory of Blood Transfusion Service (Amsterdam, the Netherlands). Horseradish peroxidase EC 1.11.1.7 type I (HRP), was obtained from Sigma Chemical Company (St. Louis, MO, USA); bovine α-thrombin from Leo Pharmaceutical Products (Ballerup, Denmark); Fura-2/AM from Molecular Probes (Eugene, OR, USA); 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP) and Sp-5,6-DCl-cBiMPS from Biolog Life Science Institute (Bremen, Germany); Prostaglandin E^1 from P-L Biochemicals. Inc. (Milwaukee, USA); ionomycin from Calbiochem Corporation (La Jolla, CA, USA); Rhodamine-labeled Ulex europaeus lectin I from Vector Laboratories Inc. (Burlingame, CA, USA); Monoclonal antibody to SMC actin (ASM-1) was obtained from Sanbio Ltd. (Uden, The Netherlands); Monoclonal antibodies to von Willebrand Factor, Fluorescein-conjugated swine Immunoglobulins (Ig) to rabbit Ig and Fluorescein-conjugated rabbit Ig to mouse Ig were obtained from Dakopatts (Denmark); Monoclonal antibody to CD31 (PECAM-1) was a kind gift from Dr. J. van Mourik (CLB, Amsterdam, The Netherlands). Purification of cGMP-PK from bovine lung and the preparation of specific antibodies against it has been described previously.[28] Antibodies against VASP and cGMP-PK II were raised as described.[24,29] Rainbow™ Molecular mass markers for SDS-PAGE were purchased from Amersham Life Sciences.

Isolation and culture of endothelial cells

Human umbilical vein EC were isolated by the method of Jaffe et al.[30] and characterized as described previously.[31] Isolation and characterization of human EC from the aorta, iliac vein and artery and foreskin microvasculature were performed as earlier described.[32,33] The blood vessels of human origin were obtained according to the guidelines of the Institutional Review Board of the University Hospital Leiden. Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C under 5% CO_2/95% air. Medium was renewed every other day. Confluent EC monolayers were released with trypsin-EDTA and subcultured on fibronectin-coated dishes or filters. For the evaluation of the presence of cGMP-PK, the phosphorylation of VASP, $[Ca^{2+}]_i$ measurements and the barrier function human aorta EC (after 2 to 9 passages), iliac vein EC (after 8 passages), umbilical vein EC (after 1 and 2 passages) and foreskin microvascular EC (after 6 to 10 passages) were used. Endothelial cells of aorta, iliac artery and umbilical vein were also used immediately after isolation to determine the presence of cGMP-PK.

Evaluation of the barrier function

Endothelial cells cultured on filters were used between 4 and 6 days after seeding. Exchange of macromolecules through the endothelial monolayers was investigated by assay of the transfer of

horseradish peroxidase (HRP). Passage of HRP through human endothelial cell monolayers was performed as described previously.[5,34] Briefly, endothelial cell monolayers were cultured on porous membranes (fibronectin-coated filters of the Transwell™ system; 0.33 cm^2, 3 μm pore size) to form a tight monolayer. Before the start of the experiment, cells were incubated for one hour in Medium 199 with albumin. In pretreatment, the cells were incubated for 15 minutes with 8-pCPT-cGMP (100 μM) in the upper and lower compartment. At the start of the experiment, 5 μg/ml horseradish peroxidase in Medium 199 with albumin was added to the upper compartment of the Transwell™ system in the presence or absence of thrombin (1 U/ml). Samples were taken from the lower compartment (at the other side of the endothelial monolayer) at various time intervals, and an equal volume of Medium 199 containing albumin was re-added to this lower compartment. Cells were kept at 37°C under 5% CO_2/95% air. All passage experiments were performed in triplicate. The concentration of HRP was derived from the HRP activity in each sample with peroxide and tetramethyl benzidine (TMB), as substrate and expressed as ng passed per cm^2 in a certain time interval.

Measurement of the intracellular calcium ion concentration

Endothelial cells were cultured on 1.5 cm^2 glass coverslips and loaded with Fura-2 by incubation with 2 μM Fura-2/AM for 45 min at 37°C in Medium 199 containing 1% albumin with or without 8-pCPT-cGMP (100 μM; 15 min preincubation). Then, the cells were washed with Tyrode buffer. The coverslips were mounted in a holder and placed in a quartz cuvet, containing 1.2 ml Tyrode buffer. Fura-2 fluorescence was continuously measured, before and after addition of thrombin (1 U/ml), with a Perkin Elmer LS 50B luminescence spectrometer (Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England). The mean intracellular calcium ion concentration ($[Ca^{2+}]_i$) was determined from a cell area of 0.6 cm^2 and was calculated by the equation:

$[Ca^{2+}]_i = K_d * \beta * [(R-R_{min})/(R_{max}-R)]$ in nM

in which R represents the ratio of the fluorescence values at 340 nm and 380 nm; R_{max} and R_{min} are the maximal and minimal ratio values, respectively, being determined after each experiment by addition of 1 μM ionomycin and 10 mM EGTA, respectively; β represents the ratio of the fluorescence at 380 nm of free Fura-2 and Fura-2 completely saturated with calcium; The fluorescence values were corrected for auto-fluorescence of unloaded cells; the K_d, the dissociation constant of the Fura-2-calcium complex, was assumed to be 224 nM at 37°C, according to Grynkiewicz et al.[35]

Immunocytochemistry

Glass coverslips were coated for 45 min with 1% gelatin which was cross-linked by an additional incubation of 15 min with 0.5% glutaraldehyde. The glass coverslips were 5 times washed with Medium 199. EC were seeded on the glass coverslips and at confluency washed with phosphate buffered saline (PBS), fixed for 10 min with methanol (-20°C) and 30 min incubated with PBS/20% human serum. The endothelial monolayers were then stained with anti cGMP-PK I, anti-CD31, anti-vWF and/or Ulex europeus lectin I for 30 min, washed three times 5 min with PBS and incubated with a second fluorescent-conjugated antibody. After 30 min the cells were three times 5 min washed with PBS and embedded in p-phenylenediamine (PPD). When indicated immunocytochemistry was also carried out as described by Reinhard et al.[14]

Detection of cGMP-PK and VASP by immunoblotting

Confluent monolayers of EC were washed three times with phosphate-buffered saline, and suspended in reducing SDS-PAGE sample buffer and boiled for 5 min. Samples (6 μg of protein) were separated on SDS-PAGE using a 10 % gel and transferred to nitrocellulose.[36] Blots were blocked

overnight at 4°C in 20 mM Tris/HCl, pH 7.5, 500 mM NaCl plus 0.1 % Tween-20, and incubated for 1 h at room temp with antibodies against cGMP-PK I (1:500), VASP (1:3000) and cGMP-PK II (1:3000) in the same buffer. The immuno reactive proteins were detected by the enhanced chemiluminescence method (ECL) as described by the manufacturer (Amersham Life Sciences).

RNA isolation and northern blotting

Total RNA was isolated according to the method of Chomczynski and Sacchi.[37] Formaldehyde-agarose gel electrophoresis, northern blotting and hybridization were performed as previously indicated.[38] An Eco R1 fragment from the expression vector pMM9, containing the complete coding sequence of the human cGMP-PK Iß, was used as a probe.[39]

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis as indicated in the text was performed with the Wilcoxon's rank sum test or the Mann-Whitney two sample test. Statistical significance was assumed if $p < 0.05$.

RESULTS

Identification of endothelial cells in culture.

Endothelial cells were identified by the characteristic morphology and specific endothelial cell markers: von Willebrand factor, CD31 (PECAM-1), uptake of DiI-acetylated LDL and the binding of Ulex europaeus lectin I. Discrimination between EC and possibly contaminating smooth muscle cells (SMC) was made by their different morphology and an antibody against SMC-α-actin. Only pure endothelial cell cultures, entirely free of SMC contamination, have been used in this study. Contamination of other cell types than SMC in EC cultures from large vessels is not likely, because of the mild method used for isolation of the EC (enzymatic separation of EC from the matrix without scraping). Cultures of microvascular EC from foreskin were SMC-α-actin negative, both in immunofluorescence cell preparations and cell lysates on western blot (not shown); they were free of elongated or spindle shaped cells. The endothelial cell cultures had a cobble-stone appearance and formed a barrier to solutes and macromolecules, indicated by the transendothelial electrical resistance and the permeability of tracer molecules.

cGMP-PK identification in human endothelial cells.

The presence of cGMP-PK in human EC was evaluated by immunofluorescence localization studies and by western blot analysis of cGMP-PK immunoreactivity in EC lysates. To ascertain the endothelial cell nature of the cells, immunofluorescence localization of cGMP-PK I in EC (Fig 1A; FITC-labeled) was combined with staining with rhodamine-labeled Ulex europaeus lectin I (Fig 1C), which recognizes EC, but not smooth muscle cells.

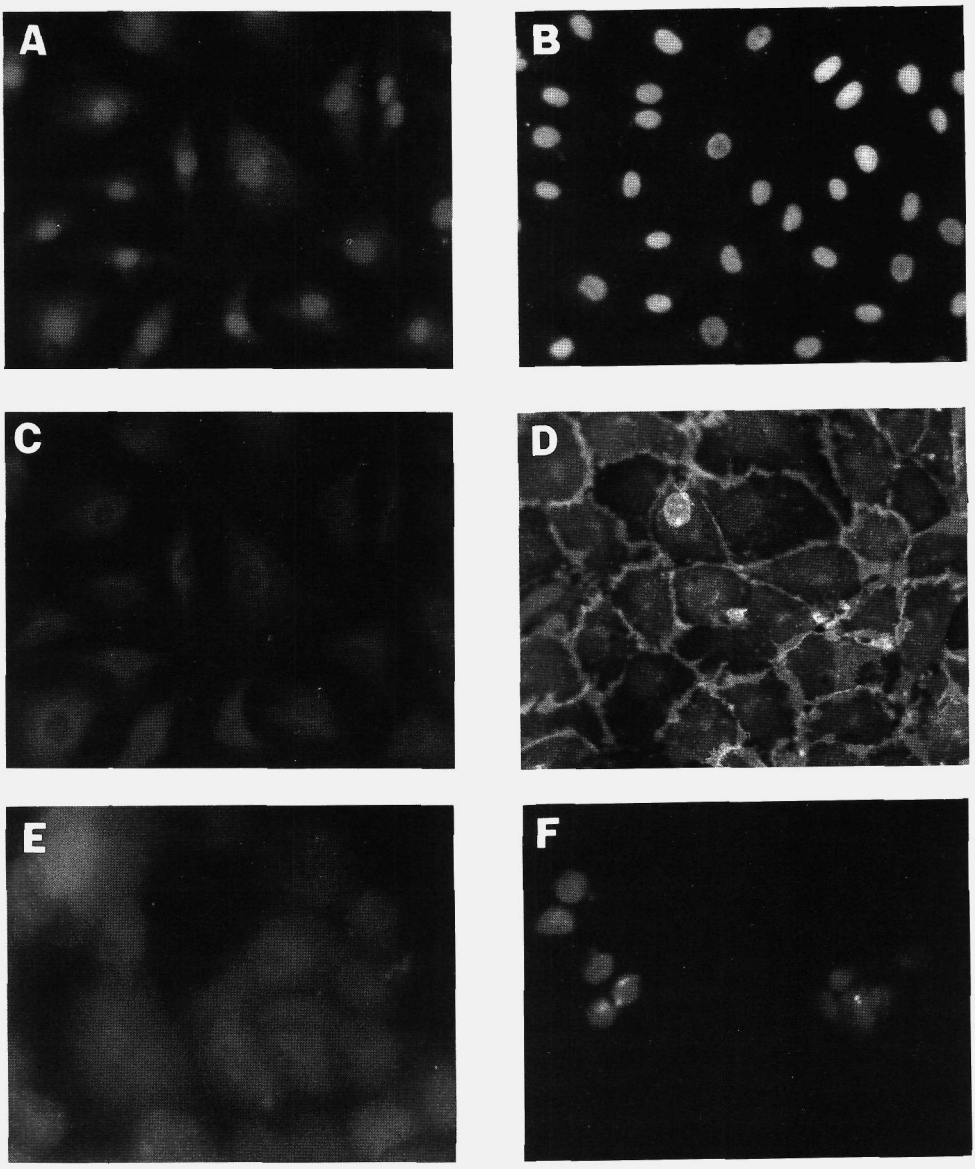

Figure 1: Immunofluorescence analysis of cGMP-PK and endothelial cell (EC) markers in aorta EC in culture and one hour after isolation. Isolation, culture and immunocytochemical staining of EC on glass-coverslips were done as described in the Materials and Methods section. Figure A-D: aorta EC in culture. A: cGMP-PK; B: negative control (aspecific staining of the nuclei with a FITC-labeled second antibody); C: Ulex europaeus lectin I (EC marker, double staining with cGMP-PK in figure A); D: EC marker CD31 (PECAM-1); Figure E-F: aorta EC one hour after isolation: E: cGMP-PK. Some of the cells are already spreading; because most of the cells are still rounded one hour after isolation, the aggregate of EC makes an unfocused appearance; F: negative control.

The endothelial cell nature of the cells was further demonstrated by the presence of CD31 at the cell-cell contacts (Fig 1D). The virtual absence of SMC was verified by establishing the absence of SMC α-actin in the cultures (not shown). cGMP-PK I was diffusively present in EC in culture derived from aorta, iliac artery and vein, and foreskin microvessels. Freshly isolated EC from 3 different aortas and an iliac artery were also positive for cGMP-PK I (Fig 1E). In contrast, EC cultured or freshly isolated from umbilical vein were negative for cGMP-PK I. SMC derived from the same umbilical veins, after additional trypsin-digestion, stained positive for cGMP-PK I and SMC α-actin (not shown).

The nature of cGMP-PK I immunoreactivity in EC lysates was further studied by western blot analysis (Fig 2). Purified cGMP-PK I from bovine lung and the lysate of a bovine aorta SMC culture were used as a reference (Fig 2, lanes 1-3 and 12). Pure endothelial cell cultures from aorta (4 different isolations), iliac vein (1 isolation) and from foreskin microvessels contained detectable amounts of cGMP-PK I, usually estimated to be about 0.15-0.5 μg cGMP-PK/mg EC protein (Fig 2, lanes 4-10). This amount is comparable with the cGMP-PK content of isolated rat aorta and bovine trachea SMC.[19,20] cGMP-PK I immunoreactivity in aorta EC remained constant in early passage EC cultures, but decreased during serial propagation of aorta EC, usually after 6 to 8 passages. This decrease resembles the loss of cGMP-PK in SMC during prolonged culture.[20] In contrast to most tissues,[11] but similar to human platelets and cultured bovine aorta EC,[40,42] cGMP-PK I was found predominantly in particulate fractions of human aorta EC extracts, and this localization did not change after activation of cGMP-PK with 8-pCPT-cGMP (not shown). However, cGMP-PK I immunoreactivity could not be detected in five freshly isolated EC preparations and in subcultures of EC from fifteen different umbilical veins (Fig 2, lane 11). The 35-40 kDa band on the Western blot appeared to be non-specific and not related to any form of cGMP-PK, as was previously observed in certain cell extracts with preimmune and antisera prepared against cGMP-PK.[40] By northern hybridization assay cGMP-PK I mRNA was detected in RNA preparations of aorta and microvascular EC, but was absent in preparations of umbilical vein EC (data not shown).

In addition to the cGMP-PK I, the presence of the exclusively membrane-bound cGMP-PK type II, which has recently been cloned from rat intestine[24] was evaluated in the EC types. Neither EC from human aorta and foreskin microvessels nor EC from umbilical vein showed a positive immunoreactivity with a cGMP-PK II-specific antibody (not shown).

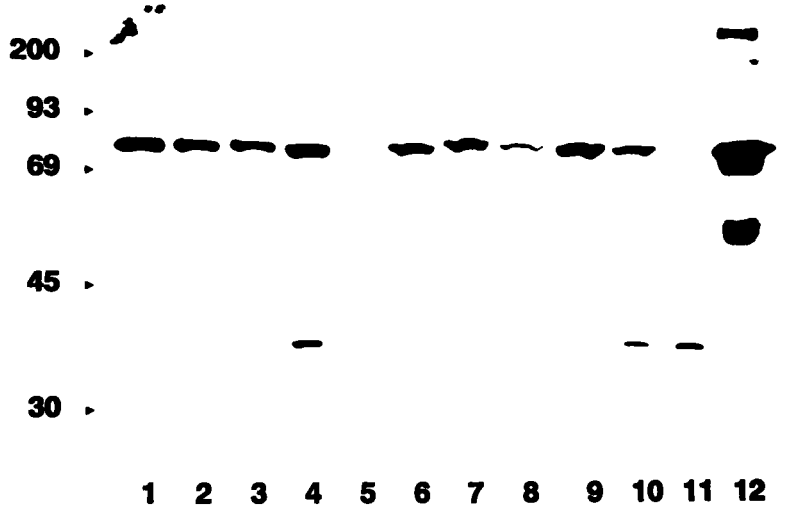

Figure 2: Autoradiogram of cGMP-PK immunoreactivity in EC lysates. Western blots of various human EC lysates (6 μg/lane) were prepared as described in the Materials and Methods section. Molecular mass markers (in kDa) are indicated on the left side of the figure.
Lanes 1-3: purified cGMP-PK standard: 4, 2 and 1 ng, respectively; Lanes 4-5: cultures of aorta EC (donor 1) after 6 and 8 passages, respectively; Lanes 6-8: cultures of aorta EC (donor 2) after 2, 5 and 9 passages, respectively; Lane 9: culture of foreskin microvascular EC after 10 passages; Lane 10: culture of iliac vein EC after 8 passages; Lane 11: culture of umbilical vein EC after 1 passage; Lane 12: culture of bovine aorta SMC after 2 passages. From these and other blots we could estimate that the various EC preparations contained about 1-3 ng cGMP-PK/6μg EC protein.

cGMP-PK phosphorylates VASP in response to 8-pCPT-cGMP.

To determine whether the cGMP-PK I in EC is active, cultures of various endothelial cell types were incubated with the cell membrane permeant cGMP-analogue 8-pCPT-cGMP, which is a selective cGMP-PK activator.[43,44] cGMP-PK activation was measured by determination of the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which is an established substrate for both cGMP-PK and cAMP-dependent protein kinase.[14,45] In confluent human umbilical vein EC VASP was localized at cell-cell contacts (Fig 3a). In subconfluent human EC, VASP was mainly found to be associated with focal contact areas and microfilaments (Fig 3b), similar to previous observations made with other cell types.[14] In all untreated endothelial cell types, VASP was predominantly present as 46 kDa form (Fig 4). After cGMP-PK activation, the 50 kDa form of VASP (phosphorylated at serine 157) was generated in intact EC from aorta, iliac vein and foreskin microvessels (Figures 4 and 5). The diminished cGMP-PK expression during subculturing of aorta EC (Fig 2) was reflected in the cGMP-PK activity: VASP phosphorylation after addition of 8-pCPT-cGMP to the cells

disappeared after prolonged culture (Fig 4). VASP phosphorylation induced by 8-pCPT-cGMP was not observed in umbilical vein EC, in accordance with the absence of cGMP-PK immunoreactivity in these cells. However, VASP phosphorylation can occur in umbilical vein EC in response to cAMP-dependent protein kinase activation by PGE_1 and Sp-5,6-DCl-cBiMPS (Fig 6).

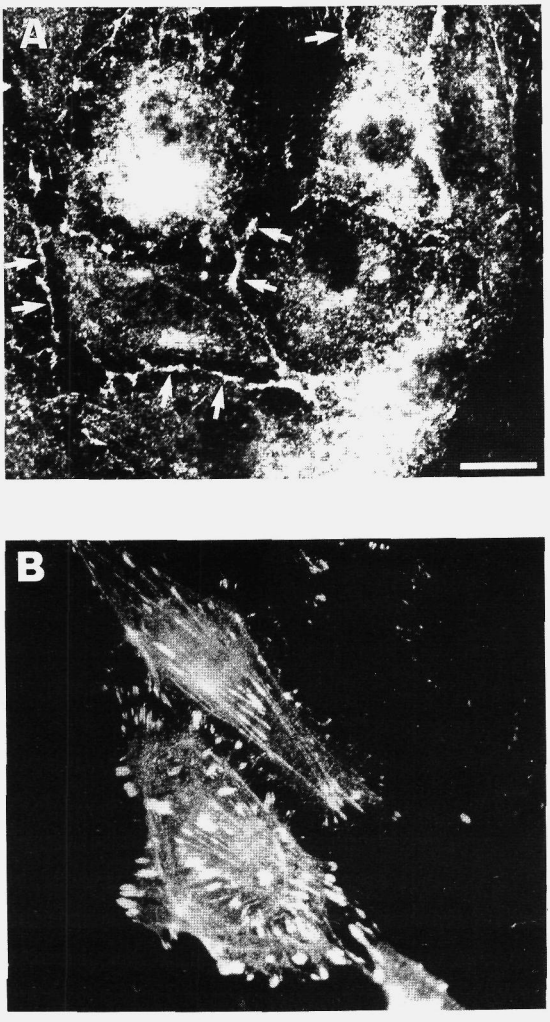

Figure 3: Immunocytochemical staining of human umbilical vein EC in culture by a monospecific VASP antiserum. A: Arrows indicate the localization of VASP at cell-cell contacts of confluent primary cultures. B: In subconfluent cells strong immunoreactivity is found to be associated with stress fibers and focal attachment sites. Experimental procedures and antibodies used were essentially as described by Reinhard et al.[14] Bar: 10 μm.

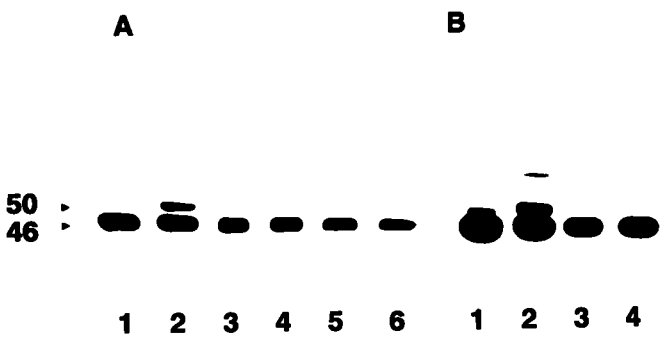

Figure 4:. Autoradiogram of VASP immunoreactivity in aorta EC lysates analyzed on western blot: cGMP-PK activation by 8-pCPT-cGMP induces phosphorylation of VASP. EC lysates were prepared for western blot as described in the Materials and Methods section. The apparent molecular masses of the unphosphorylated (46 kDa) and the phosphorylated (50 kDa) VASP forms are indicated. Figure A: aorta EC (donor 2), lanes 1, 3 and 5 untreated cells; lanes 2, 4 and 6 cells incubated for 30 min with 100 μM 8-pCPT-cGMP. The preparations were made for confluent cells after 5 passages (lanes 1-2), after 7 passages (lanes 3-4) and 9 passages (lanes 5-6). Figure B: aorta EC (donor 1), lanes 1 and 3 untreated cells; lanes 2 and 4 cells incubated for 30 min with 100 μM 8-pCPT-cGMP: lanes 1-2 after 6 passages; lanes 3-4 after 8 passages.

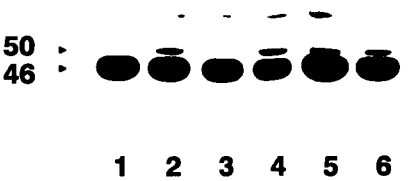

Figure 5: Autoradiogram of VASP immunoreactivity in iliac vein, foreskin microvascular and umbilical vein EC lysates analyzed on western blot: cGMP-PK activation by 8-pCPT-cGMP induces phosphorylation of VASP. EC lysates were prepared for western blot as described in the Materials and Methods section. The molecular masses of the unphosphorylated (46 kDa) and the phosphorylated (50 kDa) VASP forms are indicated. Lanes 1, 3 and 5: untreated cells, lanes 2, 4 and 6: cells incubated for 30 min with 100 μM 8-pCPT-cGMP. Lanes 1-2: iliac vein EC after 8 passages; lanes 3-4 foreskin microvascular EC after 10 passages; lanes 5-6: umbilical vein EC after 1 passage.

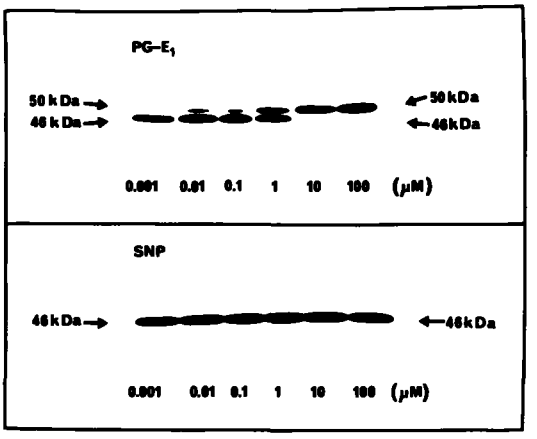

Figure 6a: Autoradiographs demonstrating the effects of prostaglandin E_1 (PGE_1) and sodium nitroprusside (SNP) on VASP phosphorylation in cultured human umbilical vein EC. Cells (passage 2) were incubated for 5 min with various concentrations of PGE_1 or SNP as indicated.
VASP phosphorylation was analyzed by western blots as described[29] and is indicated by the shift of VASP from the 46 kDa to the 50 kDa form.

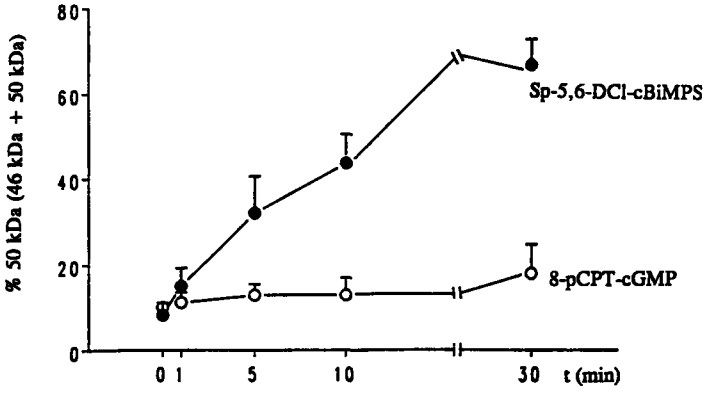

Figure 6b: Quantitative analysis of VASP phosphorylation in cultured human umbilical vein EC incubated with 500 μM Sp-5,6-DCl-cBiMPS or 200 μM 8-pCPT-cGMP for the time periods indicated. VASP phosphorylation was analyzed by western blots and is expressed as the appearance of 50 kDa VASP as percentage of total VASP (46 kDa + 50 kDa forms). Data represent the mean ± SD of three separate experiments.

Cellular consequences of cGMP-PK activation.

Elevation of intracellular cGMP attenuates thrombin-induced increase in endothelial permeability.[7,10] We used 8-pCPT-cGMP to selectively activate cGMP-PK, without affecting cGMP-regulated phosphodiesterases. Thrombin increased endothelial permeability (passage of horseradish peroxidase) in aorta, umbilical vein and foreskin microvascular EC monolayers (Fig 7A-C), which was associated with a transient increase of intracellular calcium ($[Ca^{2+}]_i$; Fig 7D-F). Preincubation with 8-pCPT-cGMP reduced the thrombin stimulated permeability in aorta EC to 59 ± 6% (mean ± SEM of 6 experiments done in triplicate, $p<0.05$; Fig 7A) in foreskin microvascular EC to 45 ± 4% (3 experiments; Fig 7B). However, the cGMP-PK activator had no effect on the permeability through umbilical vein EC monolayers: 112 ± 16% (4 experiments; Fig 7C) and 89 ± 18% (8 experiments) with 100 μM and 1 mM 8-pCPT-cGMP, respectively. Similarly, the thrombin-induced $[Ca^{2+}]_i$ elevation was inhibited by 8-pCPT-cGMP in aorta and foreskin microvascular EC (the $[Ca^{2+}]_i$ peak value decreased from 754 ± 161 to 264 ± 50 nM in aorta EC ($p = 0.002$, 10 experiments; Fig 7D) and from 791 ± 106 to 410 ± 103 nM in foreskin microvascular EC ($p = 0.05$, 5 experiments; Fig 7E)), but was not significantly affected in umbilical vein EC (from 796 ± 83 to 649 ± 67 nM, 16 experiments; Fig 7F).

Whereas activation of cGMP-PK had no effect on the permeability of umbilical vein EC, activation of cAMP-PK by PGE_1 reduced both basal and thrombin-induced endothelial permeability in vitro (Figure 8). This agrees with previous studies with the stable prostacyclin-analogue iloprost.[6,41] The effect of cAMP-PK activation did not depend on the $[Ca^{2+}]_i$, because elevation of the cellular cAMP concentration had no effect on the $[Ca^{2+}]_i$ in these cells (not shown).

DISCUSSION

Results reported here demonstrate that endothelial cells (EC) from adult human arteries and vein and from foreskin microvessels, but not those of human umbilical vein, contain active cGMP-dependent protein kinase type I (cGMP-PK I). The selective cGMP-PK activator 8-pCPT-cGMP caused VASP phosphorylation, reduced thrombin-stimulated cytoplasmic calcium ion ($[Ca^{2+}]_i$) accumulation, and reduced thrombin-induced increase in permeability. These effects were restricted to cGMP-PK-containing EC.

cGMP-PK was originally not found in cultured bovine pulmonary artery and aorta EC,[46] but Mac-Millan-Crow et al.[42] and Diwan et al.[47] recently reported the presence of cGMP-PK I in extracts of bovine aorta and rat pulmonary microvascular EC, respectively. The different results in these studies may be due to a culture phenomenon, because the cGMP-PK contents

decreases below detection level during serial propagation of human aorta EC (Fig 2). We found cGMP-PK in a range of 0.15-0.5 μg/mg cellular protein in adult artery and vein EC and in microvascular EC, which is somewhat less than found in isolated human platelets,[48] but similar to the level detected in SMC in vitro.[19,20] Interestingly, human umbilical vein EC, which have many properties in common with postcapillary venule EC, did not contain cGMP-PK, neither in culture nor immediately after isolation from the native blood vessel.

The differences in cGMP-PK content of various types of EC may largely determine the effect of cGMP-enhancing agents on the endothelium of a particular type of blood vessel. This suggestion is supported by the observation that a thrombin-induced increase in $[Ca^{2+}]_i$ and endothelial permeability was reduced by 8-pCPT-cGMP in cGMP-PK-containing aorta and microvascular EC, whereas this cGMP-analogue was ineffective in cGMP-PK-negative umbilical vein EC (Fig 7). Elevation of $[Ca^{2+}]_i$ has been associated with an increase in endothelial contraction and permeability.[2,3,5,23,49,50,51] The observed cGMP-PK-mediated reduction of endothelial permeability may therefore be a direct consequence of the reduction of the thrombin-induced $[Ca^{2+}]_i$ rise. In this respect regulation of EC contraction, which underlies the increase in intercellular permeability, appears to be rather similar to the regulation of SMC contraction and platelet activation.[16,52] However, SMC contraction may also be modulated by mechanisms other than $[Ca^{2+}]_i$ regulation.[53] Therefore, we can not exclude the possibility that cGMP-dependent phosphorylation also contributes by additional Ca^{2+}-independent mechanisms to a change in the interaction of actin filaments with myosin or cell-cell contact areas and hence influences endothelial permeability. One possible additional mechanism could be cGMP-PK mediated VASP phosphorylation.

Our data show that the cGMP-PK-I in EC is active and phosphorylates VASP. Very recent data suggest that VASP phosphorylation does not directly regulate phopholipase C activity or Ca^{2+} mobilization, but appears to be involved in regulating the actin filament sytem, possibly via profilin, and proteins associated with cell-matrix (focal adhesions) and cell-cell contacts.[15,39,54] In this context, our finding that VASP is localized at cell-cell contact areas of confluent EC, while it is predominantly present in focal adhesion sites in subconfluent EC, is of interest. This spacial distribution has much similarity with the distribution of cadherins in EC[55] and suggests that VASP may participate in the organisation of cell-cell contacts of the adherens type. Further studies are clearly necessary to elucidate whether VASP is involved in the maintenance of the endothelial barrier function and whether the phosphorylation of VASP contributes to the regulation of endothelial permeability. If this would be the case, VASP may act as a convergence point for cGMP and cAMP, which both induce a phosphorylation of VASP at three distinct sites ([45]; this study), and both can inhibit an agonist-induced increase in permeability.[10,56]

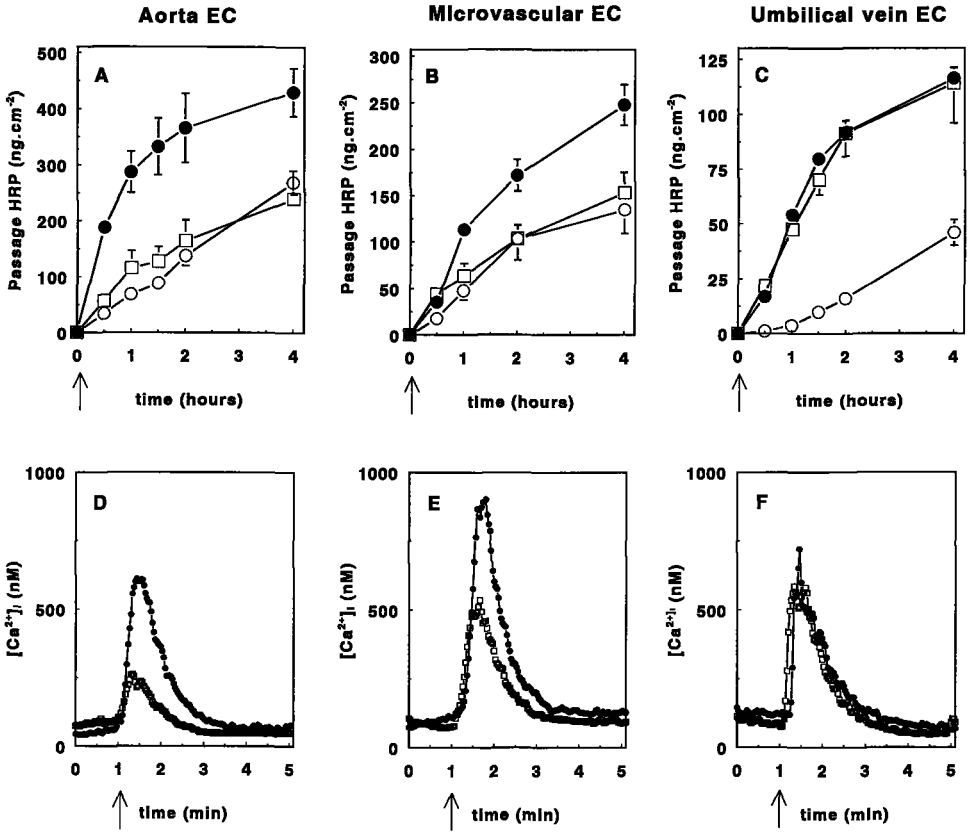

Figure 7: Thrombin-induced increase of endothelial permeability and $[Ca^{2+}]_i$ in the absence or presence of 8-pCPT-cGMP. The endothelial permeability was measured by the passage of horseradish peroxidase through EC monolayers and the $[Ca^{2+}]_i$ was determined in Fura-2 loaded EC, as described in the Materials and Methods section. Figure A: Passage of horseradish peroxidase through aorta EC monolayers under control conditions (-○-), after addition of 1 U/ml thrombin without (-●-) and with (-□-) a preincubation of 100 μM 8-pCPT-cGMP. The arrow indicates the time point of thrombin addition; Figure B/C: Similar as described for panel A for foreskin microvascular EC and umbilical vein EC, respectively; Figure D: $[Ca^{2+}]_i$ in aorta EC after addition of thrombin (indicated by an arrow on the x-axis) without (-●-) and with (-□-) a preincubation of 8-pCPT-cGMP; Figure E/F: Similar as described for panel D for foreskin microvascular EC and umbilical vein EC, respectively.

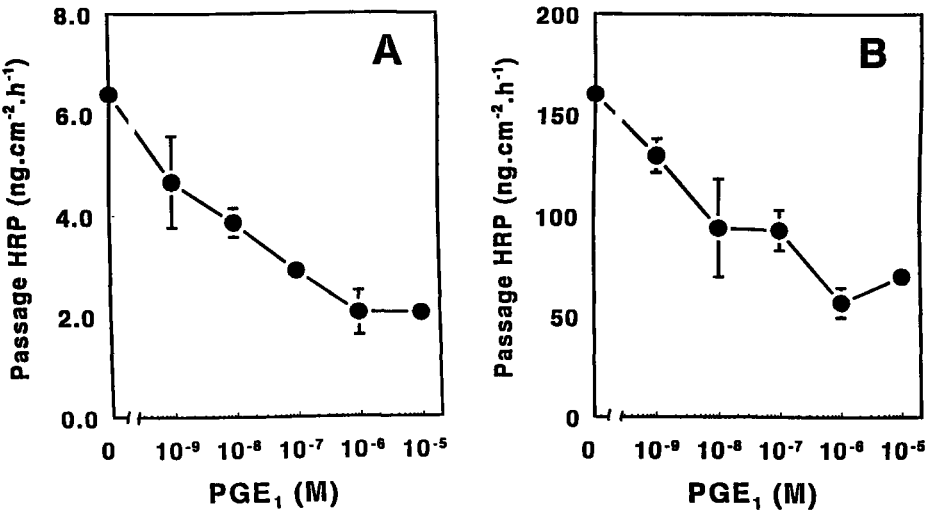

Figure 8: Effect of prostaglandin E_1 (PGE_1) on the basal and thrombin-stimulated passage of horseradish peroxidase (HRP) through human umbilical vein EC monolayers evaluated over a two hours incubation period. A: Passage of HRP under basal condition. B: Passage of HRP under thrombin (1 U/ml)-stimulated condition. Values are mean ± SEM of triplicate determinations. Similar results were obtained with another tracer molecule (FITC-labeled 38.9 kDa dextran).

In addition to endothelial permeability, the synthesis and/or release of a number of EC products involved in vasoregulation and hemostasis is enhanced by vasoactive substances, at least in part via an increase in $[Ca^{2+}]_i$. They comprise the release of nitric oxide (NO)[57] and prostacyclin,[58] the acute release of tissue-type plasminogen activator and von Willebrand factor[59] and the intracellular production of platelet activating factor.[60] Elevation of cGMP by administration of atrial natriuretic peptide (ANP) or 8-bromo-cGMP reduced the acute release of t-PA induced by vasoactive substances in the perfused rat hindleg.[61] The same mediators inhibited thrombin-induced release of endothelin-1 by rat aorta EC.[62] Furthermore, the production of NO has been reported to counterregulate its own production,[63] probably because it reduces the accumulation of $[Ca^{2+}]_i$ via its activation of guanylyl cyclase.

Our data suggest that the expression of cGMP-PK I, VASP and probably additional substrates may be important in the regulation of various EC functions. The presence of cGMP-PK in combination with NO synthase, and soluble and natriuretic peptide receptor-

linked guanylyl cyclase equips the EC with a potent (counter-)regulatory mechanism for maintaining hemostasis.

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CHAPTER 6

EFFECT OF PROTEIN KINASE C ACTIVATION ON THE PERMEABILITY OF HUMAN ENDOTHELIAL CELL MONOLAYERS

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ABSTRACT

The role of PKC activation in the permeability of human endothelial cell (EC) monolayers was investigated. The protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) reduced the permeability of dextran (38.9 kDa) through human umbilical vein EC monolayers in a concentration-dependent way with a maximal reduction at 10 nM. The PMA-induced decrease in permeability was prevented by the PKC-inhibitor Ro31-8220. Addition of thrombin increased the endothelial permeability tenfold, which was 50% inhibited by 10 nM PMA. Thrombin-stimulated permeability was not affected by the PKC-inhibitors Ro31-8220 (1 μM) and calphostin C (100 nM). Effects of PMA under thrombin-stimulated conditions were mimicked by 1-oleoyl-2-acetylglycerol (OAG) and the Ca^{2+}-dependent PKC activator thymeleatoxin (Tx). The observed PKC effects were not specific for umbilical vein EC, since PKC activation also reduced the permeability of human foreskin microvascular EC monolayers. Thrombin induced an increase in the cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$), which was reduced by preincubation of the cells with PMA. Intracellular cAMP, cGMP and ATP concentrations were not affected by PMA. After preincubation of umbilical vein and microvascular EC with PMA, the stress fibres consisting of F-actin, and the co-localization of F-actin and non-muscle myosin disappeared. We conclude that activation of Ca^{2+}-dependent PKC by 10 nM PMA decreases the permeability of human EC monolayers. PKC activation reduces thrombin-stimulated $[Ca^{2+}]_i$ elevation and inhibits actin-non-muscle myosin interaction, which both may contribute to a reduced contractile response of human EC. The observed increase in permeability at higher PMA concentrations suggests that multiple counteracting effects of PKC activation affect endothelial permeability.

INTRODUCTION

The exchange of fluid, nutrients and macromolecules between blood and tissues is regulated by the endothelium. Endothelial cells (EC) actively respond to vasoactive substances, such as histamine, platelet activating factor, bradykinin and thrombin. These agents cause a contraction of the EC of postcapillary venules and subsequently induce leakage of fluids and proteins through the endothelial monolayer.[9,35] The contractile response involves actin-non-muscle myosin interaction, which requires calcium, calmodulin and ATP and phosphorylation of myosin light chain (MLC).[38,48,60,61] This process is regulated by the MLC kinase and cAMP- and cGMP-dependent protein kinases.[3,6,14,29,38,53,61] Furthermore, a number of *in vivo* and *in vitro* experiments suggest a regulatory role of protein kinase C (PKC) in endothelial contraction and permeability. Prevention by PKC-inhibitors of bradykinin and plant lectin-induced leakage in the rat- and hamster microvasculature have suggested a role for PKC activation in increased endothelial permeability.[39,42]

Endothelial cells *in vitro* have been proved to be helpful to provide biochemical information regarding processes involved in the regulation of endothelial permeability. PKC activation by phorbol 12-myristate 13-acetate (PMA) at high concentrations (0.1-1 μM) increased the permeability of bovine- and porcine EC monolayers.[4,19,33,43,52] Endothelial cell monolayers challenged with H_2O_2 became more prone to leak, which was prevented by the PKC inhibitors H7 and calphostin C.[49] Similarly, the thrombin-induced increase in endothelial permeability was reduced by H7.[32,33] From these data it has been suggested that PKC activation is a requirement for endothelial cell contraction induced by thrombin or H_2O_2.[33,49] On the other hand, Yamada et al[62] observed a reduction in endothelial permeability by a moderate PMA concentration in human umbilical vein endothelial cells (HUVEC). To evaluate further the role of PKC activation in the regulation of the permeability of human EC, we have used HUVEC and human foreskin microvascular EC cultured as tight monolayers on porous filters.[14,27,29] We report here the effects of specific PKC activators and inhibitors on basal and thrombin-stimulated permeability and on the cytoplasmic concentrations of calcium ions and cyclic nucleotides. Our data suggest multiple effects of PKC activation on the permeability of human EC monolayers.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics from Corning (Corning, NY, USA) or Costar (Cambridge, MA, USA); and Transwells (diameter 0.65 cm, pore size 3 μm) from Costar. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al.[34] Human serum was obtained from the local blood bank and was prepared from fresh blood taken from healthy donors; the sera were pooled, and stored at 4°C. Newborn calf serum (NBCS) was obtained from GIBCO (Grand Island, NY, USA) and heat-inactivated before use (30 min, 56°C). Pyrogen-free human serum albumin (HSA) was purchased from the Central Laboratory of Blood Transfusion Service (Amsterdam, the Netherlands). Fluorescein isothiocyanate-labeled dextran (dextran-FITC) with molecular mass 38900 D, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4α-PDD) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) was obtained from Sigma Chemical Company (St. Louis, MO, USA); 12-deoxyphorbol-13-phenylacetate (DOPP), DOPP-20-acetate (DOPPA), thymeleatoxin (Tx) and calphostin C were obtained from LC laboratories (Nottingham, UK). Ro31-8220 was a kind gift from Dr PA Brown, Roche Products Ltd (UK); bovine α-thrombin was obtained from LEO Pharmaceutical Products (Ballerup, Denmark); human atrial natriuretic peptide-(99-128) from Bissendorf peptide GmbH; forskolin from Hoechst (La Jolla, CA, USA); isobutyl-methyl-xanthine (IBMX) from Janssen Chimica (Beerse, Belgium); fura 2-AM and rhodamine phalloidin from Molecular Probes (Eugene, OR, USA); anti-myosin IgG (non-muscle) from Sanbio (Uden, Netherlands); Rabbit anti-mouse IgG-FITC from Dakopatts (Denmark).

Isolation and culture of endothelial cells

Human EC from umbilical vein and foreskin microvessels were isolated and characterized as described previously.[11,26,56,57] The blood vessels of human origin were obtained according to the guidelines of the Institutional Review Board of the University Hospital Leiden. Cells were cultured on fibronectin-coated dishes in Medium 199 supplemented with 10% human serum, 10% NBCS, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin, 100 U/ml penicillin and 0.1 mg/ml streptomycin and kept at 37°C under 5% CO_2/95% air. Experiments were performed with confluent monolayers of EC from umbilical vein (first passage) or foreskin mirovessels (fifth to twelfth passage), which were released with trypsin-EDTA and seeded in high density on fibronectin-coated polycarbonate filters of the Transwell™ system, on glass-slides or dishes and cultured as previously described.[14,27,28]

Evaluation of the barrier function

Endothelial cells cultured on filters were used 5 days after seeding. Exchange of macromolecules through the endothelial monolayers was investigated by assay of the transfer of dextran-FITC (1 mg/ml) as described previously.[14,28] Briefly, endothelial cell monolayers were cultured on porous membranes (0.33 cm^2; 3 μm pore size) to form a tight monolayer. The cells were incubated in M199 with 1 % albumin one hour before and during the experiment. Dextran-FITC was added to the upper compartment of the Transwell™ system in the presence or absence of PMA, DOPP, DOPPA, Tx, OAG, Ro31-8220, calphostin C and/or thrombin. Samples were taken from the lower compartment and an equal volume of Medium 199 with 1% albumine was re-added to this lower compartment. Cells were kept at 37°C under 5% CO_2/95% air. Passage experiments were performed in duplicate

or triplicate. The concentration of the dextran-FITC in samples of under and upper compartiment was measured by a Perkin Elmer LS 50B luminescence spectrometer.

Measurement of intracellular cyclic nucleotide concentrations

The cyclic nucleotide concentrations in human EC were determined by radio-immunoassay as described by Draijer et al.[14] Briefly, to determine the concentrations of cAMP and cGMP the cells were preincubated for 15 min with the phosphodiesterase inhibitor IBMX (1 mM) and subsequently with the concentrations of PMA, thrombin, ANP or forskolin indicated. After 15 min the experiment was terminated by the addition of 3.5% perchloric acid. The cell lysates were neutralized by $KHCO_3$, dried under a stream of N_2-gas and kept at -20°C until determination by radio-immunoassay (Amersham, Amersham, UK).

Measurement of intracellular calcium ion concentration

Endothelial cells were cultured on 1.5 cm^2 glass coverslips and loaded with fura-2 by incubation with 2 μM fura-2/AM for 45 min at 37°C in Medium 199 containing 1% albumin. Then, the cells were washed with Tyrode buffer. The coverslips were mounted in a holder and placed in a quartz cuvet, containing 1.2 ml Tyrode buffer. Fura-2 fluorescence was continuously measured, before and after the addition of thrombin (1 U/ml), with a Perkin Elmer LS 50B luminescence spectrometer (Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England). The mean intracellular calcium ion concentration ($[Ca^{2+}]_i$) was determined from a cell area of 0.6 cm^2 and was calculated by the equation:

$$[Ca^{2+}]_i = K_d * \beta * [(R-R_{min})/(R_{max}-R)] \quad \text{in nM}$$

in which R represents the ratio of the fluorescence values at 340 nm and 380 nm; R_{max} and R_{min} are the maximal and minimal ratio values, respectively, being determined after each experiment by addition of 1 μM ionomycin and 10 mM EGTA, respectively; β represents the ratio of the fluorescence at 380 nm of free fura-2 and fura-2 completely saturated with calcium; The fluorescence values were corrected for auto-fluorescence of unloaded cells; the K_d, the dissociation constant of the fura-2-calcium complex, was assumed to be 224 nM at 37°C, according to Grynkiewicz et al.[18]

Immunocytochemistry

Glass coverslips were coated for 45 min with 1% gelatin which was cross-linked by an additional incubation of 15 min with 0.5% glutaraldehyde. The glass coverslips were washed 5 times with Medium 199. EC were seeded on the glass coverslips and at confluency, if indicated, incubated with 1U/ml thrombin for one hour in medium 199 with 1% albumin, and then washed with medium 199, fixed for 10 min with paraformaldehyde, permeabilized with Triton-X100 for 2 min and washed again. The endothelial monolayers were then stained with antibodies raised against non-muscle myosin or rhodamine phalloidine for 30 min, washed three times for 5 min with phosphate buffered saline (PBS) and incubated with a second fluorescent-conjugated antibody. After 30 min the cells were washed three times for 5 min with PBS and embedded in p-phenylenediamine (PPD).

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analysis as indicated in the text was performed with the Wilcoxon's rank sum test. Statistical significance was assumed if $p < 0.05$.

RESULTS

Dual effect of protein kinase C activation by phorbol ester on the permeability of human EC monolayers

A 38.9 kDa FITC-labelled dextran was used to evaluate the permeability of human umbilical vein EC (HUVEC) monolayers. The protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) reduced the basal endothelial permeability when added in a low nanomolar range, whereas a concentration of 0.1 μM PMA reversed this effect. The non-tumour analogue 4α-PDD had no effect (Fig 1A). At a concentration of 10 nM, PMA reduced the passage of FITC-dextran from 0.92 $\pm$ 0.19 $\mu g.cm^{-2}$ to 0.69 $\pm$ 0.18 $\mu g.cm^{-2}$ during the first hour after PMA addition (mean $\pm$ SEM, 12 cultures, $p<0.001$). The maximal decrease in permeability induced by 10 nM PMA was reached after one hour and then remained constant for at least 4 hours (Fig 1B). The PKC inhibitor Ro31-8220 was used to obtain additional evidence that the effect of PMA reflected activation of PKC. Ro31-8220 (1 μM) prevented the effect of 10 nM PMA on the endothelial permeability (Table 1). Ro31-8220 tended to increase the endothelial permeability but this did not reach significance.

Activation of the thrombin receptor by thrombin causes activation of multiple signal transduction pathways including activation of PKC. The thrombin-induced increase in the permeability of bovine pulmonary artery EC monolayers has been demonstrated to require PKC activity. Because we observed that concentrations up to 10 nM PMA reduced the permeability of HUVEC and 10 nM PMA in our hands is a stronger inducer of PKC-dependent gene (t-PA) activation than 1 U/ml thrombin, we investigated the effects of 10 nM PMA and inhibition of PKC activity in the permeability of HUVEC monolayers. Thrombin (1U/ml) increased the permeability of HUVEC monolayers from 0.92 $\pm$ 0.19 to 11.5 $\pm$ 2.4 $\mu g.h^{-1}.cm^{-2}$ (12 cultures, $p<0.001$). When PMA (10 nM) and thrombin were simultaneously added to the endothelial monolayers no additional increase in the permeability was observed. A representative time course of the effect of 10 nM PMA and thrombin is given in Figure 1C. However, after prolonged incubation periods the presence of PMA reduced the thrombin-enhanced permeability. This reduction was 15% after 30 min and reached a maximum of 50% after 120 min (Fig 1D). Additionally, when HUVEC were preincubated for 15 min or 120 min with 10 nM PMA, the thrombin-induced increase in permeability was even more effectively counter-regulated (Fig 1D). Apparently, activation of PKC before the addition of thrombin rapidly prevented the contractile response of EC, whereas it was much less effective once thrombin activation of the cells had occurred.

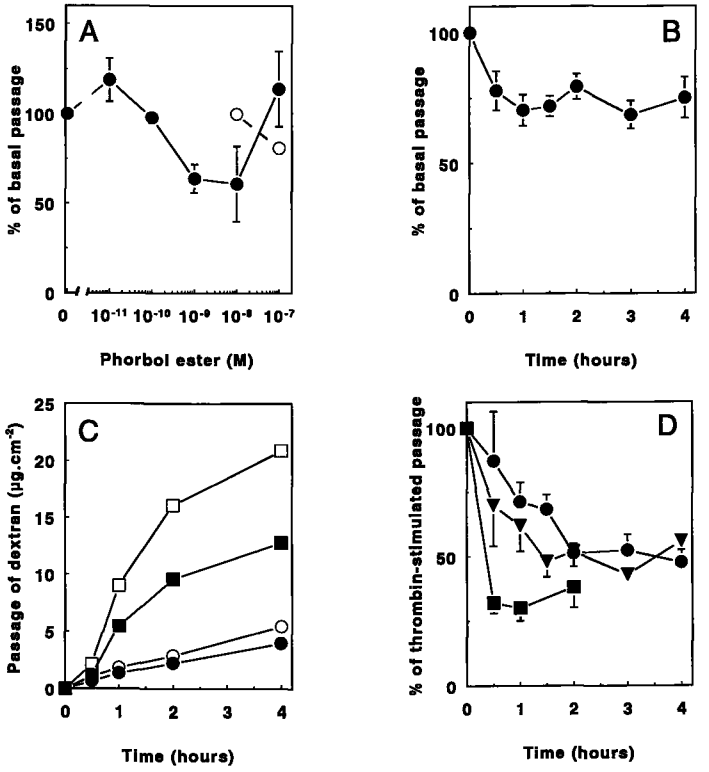

Figure 1: Passage of dextran through human umbilical vein endothelial cell (HUVEC) monolayers in the presence of PMA or thrombin (1 U/ml). A: Basal passage of dextran after one hour incubation in the presence of various PMA (●) and 4α-PDD (○) concentrations. Values are % of control (1.48 ± 0.29 $\mu g.h^{-1}.cm^{-1}$), mean ± SEM of three cultures. B: Time course of basal passage of dextran in the presence of 10 nM PMA. Values are % of control (0.92 ± 0.19 $\mu g.h^{-1}.cm^{-1}$), mean ± SEM of 12 cultures. C: Representative experiment of basal- and thrombin-stimulated passage of dextran in the absence or presence of PMA. The basal permeability (○) reduced after addition of 10 nM PMA (●); permeability increased after addition of thrombin (□), which was partly prevented by simultaneous incubation with 10 nM PMA (■). D: Time course of thrombin-stimulated passage of dextran in the presence of 10 nM PMA. Reduction of the endothelial permeability by PMA develops slowly. Values are % mean ± SEM of thrombin-stimulated passsage of EC cultures preincubated with PMA for 0 (●, 4 cultures), 15 (▾, 6 cultures) or 120 min (■, 5 cultures).

Subsequently, the PKC inhibitors Ro31-8220 and calphostin C were used to evaluate the role of PKC in the thrombin-induced increase in endothelial permeability. Ro31-8220 (1 µM) caused no significant alteration of the thrombin-stimulated permeability (Table 1).
Calphostin C (100nM) slightly increased the basal permeability from 0.82 ± 0.17 to 1.21 ± 0.36 $\mu g.h^{-1}.cm^{-2}$, but the thrombin-stimulated permeability was not affected by 15 min preincubation with calphostin C, being 7.8 ± 1.7 $\mu g.h^{-1}.cm^{-2}$ and 7.2 ± 1.4 $\mu g.h^{-1}.cm^{-2}$,

respectively (4 cultures). The PMA-induced decrease in thrombin-stimulated endothelial permeability was prevented by 15 min preincubation with Ro31-8220 (Table 1). Ro31-8220 regularly caused massive leakage after about four hours incubation (not shown). This may point to a toxic effect on the EC at prolonged incubation, but protein synthesis as determined by the incorporation of ^{35}S-methionine in proteins was not affected during this time period.

Table 1: Passage of dextran (38.9 kDa) through human umbilical vein EC monolayers in the absence or presence of PMA, Ro31-8220 and/or thrombin.

	Passage of dextran ($\mu g.h^{-1}.cm^{-2}$)	
Addition	Control	+Thrombin
None	1.30 ± 0.18 (8)	14.0 ± 3.4 (7)
Ro31-8220	2.32 ± 0.45 (8)	14.4 ± 4.9 (7)
PMA	1.01 ± 0.16* (7)	6.2 ± 1.5* (6)
PMA+Ro31-8220	2.41 ± 0.52*,# (7)	15.4 ± 2.4# (6)

Endothelial cells were incubated under basal conditions with Ro31-8220 (1 μM) 15 min prior to PMA; PMA (10 nM) or Ro31-8220 were added 15 min before thrombin (1 U/ml). The passage of dextran was determined after one hour (thrombin) or two hours (basal) incubation. Values are mean ± SEM of the number of different cultures (n); *$P < 0.05$ vs. corresponding control value; #$P < 0.05$ vs. corresponding PMA value.

Effect of various protein kinase C activators on the permeability of human umbilical vein and microvascular EC monolayers

Besides PMA, two other non-specific PKC activators, the diacylglycerol analogue 1-oleoyl-2-acetylglycerol (OAG) and 12-deoxyphorbol-13-phenylacetate (DOPP), as well as thymeleatoxin (Tx), which predominantly activates calcium-dependent PKC (PKCα,β,γ), and DOPP-20-acetate (DOPPA), a specific activator of PKCβ1, were used to evaluate the role of PKC on the endothelial barrier function. The various agonists at 10 nM concentration (OAG at 50μM) had little effect on the basal endothelial permeability; only Tx partly mimicked the effect of 10 nM PMA. In contrast, under thrombin-stimulated conditions OAG and Tx reduced endothelial permeability to a similar extent as 10 nM PMA, while DOPP and DOPPA were less effective. The effects of these agents were prevented by preincubation of

the cells with R031-8220 (Table 2; Fig 2A). DOPPA tended to increase the permeability of HUVEC monolayers at a concentration of 0.1-1 μM under both basal and thrombin-stimulated conditions (Fig 2A).

Table 2: Effect of protein kinase C activators PMA, DOPP, DOPPA, Tx and OAG on permeability of dextran through human umbilical vein EC monolayers.

	Passage of dextran (% of control)			
	Control	Ro31-8220	Thrombin	Thrombin+ Ro31-8220
Control	≡ 100	195 ± 43 (8)	≡ 100	101 ± 20 (7)
PMA (10nM)	80 ± 4* (7)	190 ± 26 (7)	63 ± 14* (6)	138 ± 27 (6)
DOPP (10nM)	105 ± 24 (5)	158 ± 40 (3)	76 ± 9 (6)	105 ± 20 (4)
DOPPA (10 nM)	87 ± 13 (7)	165 ± 37 (3)	81 ± 8* (8)	110 ± 20 (4)
Tx (10 nM)	65 ± 15 (6)	84 ± 19 (4)	58 ± 13* (7)	107 ± 30 (5)
OAG (50μM)	99 ± 23 (5)	N.D.	56 ± 6* (6)	N.D.

Ro31-8220 (1 μM) was added 15 min prior to PMA, DOPP, DOPPA, Tx or OAG. Values are mean ± SEM of percentage of control under basal- and thrombin-stimulated conditions of the number of different cultures (n), measured during a one hour incubation; N.D. not determined. *$P < 0.05$ vs control.

To investigate whether the observed effects of PKC activation on endothelial permeability was a specific feature of HUVEC or also applicable to other types of human EC, we evaluated the effects of various PKC activators on human foreskin microvascular EC. Similarly as found in HUVEC, the permeability of human foreskin microvascular EC was reduced by PMA (1 and 10 nM), in particular when the cells were stimulated by thrombin (Fig 2B). The basal permeability was not affected by other PKC activators, but 10 nM Tx and, to a lesser extent, DOPP reduced the thrombin-induced increase in permeability.

Actin-non-muscle myosin co-localization in HUVEC and human microvascular EC monolayers

Interaction between actin and non-muscle myosin is responsible for endothelial contraction and the resulting increase in permeability. Direct or indirect interference with this interaction by PKC activation may contribute to the observed effects of PMA on the endothelial permeability. Actin and non-muscle myosin were localized by fluorescence-microscopy in HUVEC and microvascular EC. Figure 3 shows these data for human

microvascular EC, which are essentially the same as for HUVEC (not shown). Actin formed under basal conditions a dense peripheral band in EC with assembled crossings between neighbouring cells. Occasionally, stress fibres across the EC were observed (Fig 3A). The actin distribution in the presence of the adenylate cyclase activator forskolin was essentially the same as under control conditions (Fig 3C). Incubation of the cells with PMA for one hour gave the actin-containing cell margins a "wrinkled" appearance, but the EC monolayer remained intact (Fig 3B). Thrombin induced a contraction of the EC, which was attended by the appearance of many stress fibres and small gaps between the cells (Fig 3G). Preincubation of forskolin diminished the number of actin stress fibres and restored the EC monolayer (Fig 3I). Preincubation of the cells with 10 nM PMA also prevented the formation of stress fibres and preserved the actin localization at the cell margins; but the cell margins obtained a "wrinkled" appearance (Fig 3H). Under basal conditions non-muscle myosin was localized both diffusively through the cell and associated with actin cables (Fig 3D). Co-localization of non-muscle myosin and actin in the cell margins and stress fibres was more pronounced after thrombin stimulation (Fig 3J). This co-localization disappeared entirely after a 1 hour incubation of the cells with forskolin (Fig 3F,L), or with 10 nM PMA (Fig 3E,K).

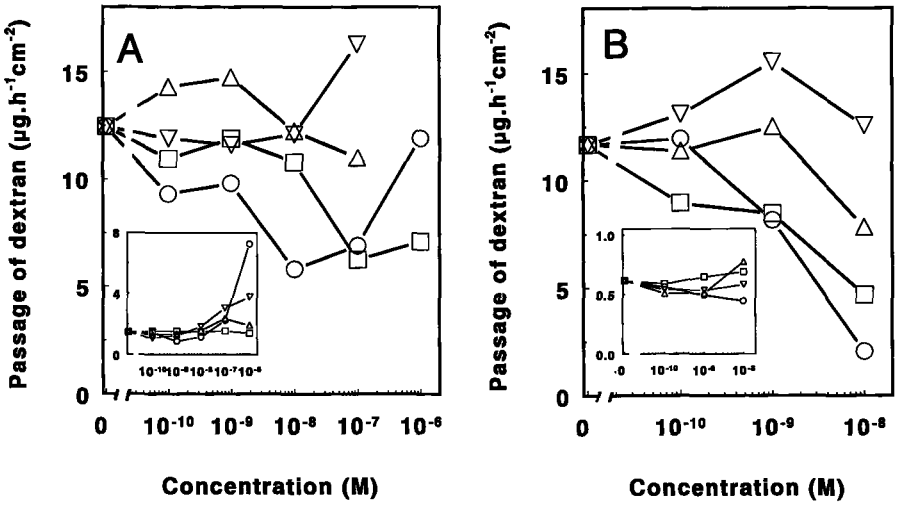

Figure 2: Passage of dextran through human EC monolayers in the presence of various concentrations of PKC-activators. A: HUVEC monolayers were incubated with PMA (○), DOPP (△), DOPPA (▽) or Tx (□) under basal condition (inset) or thrombin-stimulated condition. The passage of dextran was determined after one hour (thrombin) or two hours (basal) incubation. The PKC-activators were added in a concentration range from 10^{-10} to 10^{-6} M at t=0 under basal conditions or preincubated for 120 min under thrombin-stimulated conditions. B: like Fig 3A for foreskin microvascular EC monolayers, PKC-activators in a concentration range from 10^{-10} to 10^{-8} M.

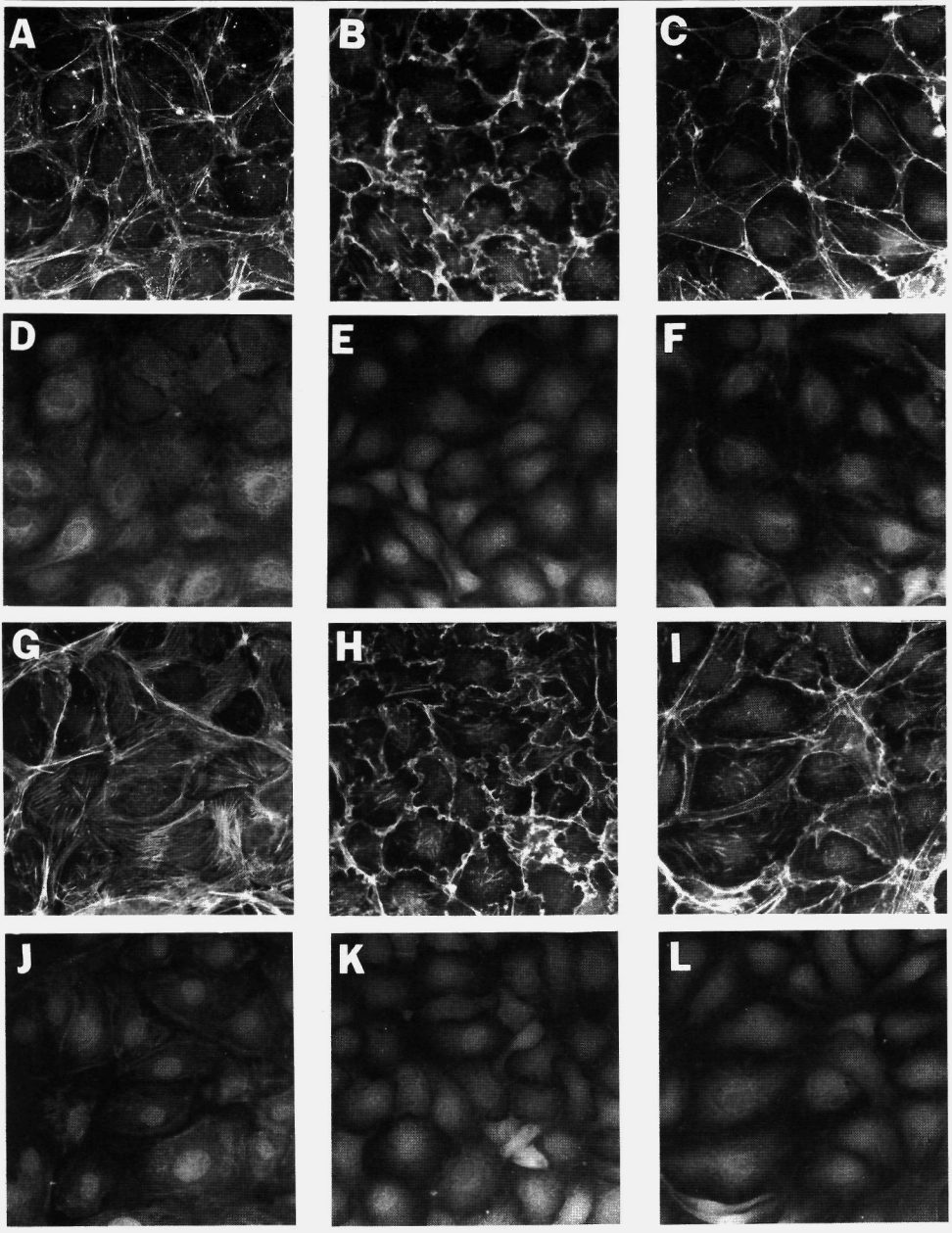

Figure 3: Immunocytochemical staining of actin and non-muscle myosin in human foreskin microvascular EC. EC were incubated with 10nM PMA or 10 μM forskolin and, if indicated, with 1U/ml thrombin for one hour. The cells were fixed with paraformaldehyde and stained with rhodamine-phalloidine and an anti-myosin monoclonal antibody as described in the Methods section. Fig A, B, C, G, H, I actin; D, E, F, J, K, L myosin. A and D, basal condition; B and E, PMA; C and F, forskolin; G and J, thrombin; H and K, thrombin+PMA; I and L, thrombin+forskolin.

Effect of PMA on basal and thrombin-enhanced concentrations of cytoplasmic calcium ions.

Because calcium-calmodulin-dependent activation of the myosin light chain kinase and subsequent phosphorylation of the myosin light chain are pivotal steps in the thrombin-induced increase of actin-non-muscle myosin interaction and endothelial permeability,[14,17,48,61] the effect of 10 nM PMA on the basal and thrombin-induced increase in cytoplasmic calcium ion concentration was evaluated. To that end, the $[Ca^{2+}]_i$ under basal and thrombin-stimulated conditions was measured with and without a 5 min preincubation of PMA in fura 2-loaded HUVEC. The basal $[Ca^{2+}]_i$ (97 ± 9 nM, 14 determinations in 4 different EC cultures) was not affected by PMA (94 ± 10 nM). The $[Ca^{2+}]_i$ in these cultures increased immediately after the addition of thrombin to 479 ± 41 nM and remained elevated after 2 min at 213 ± 14 nM (Figure 4A). Preincubation of PMA reduced the initial $[Ca^{2+}]_i$-increase and the plateau phase to 221 ± 31 nM ($P<0.05$) and 107 ± 12 nM ($P<0.01$), respectively.

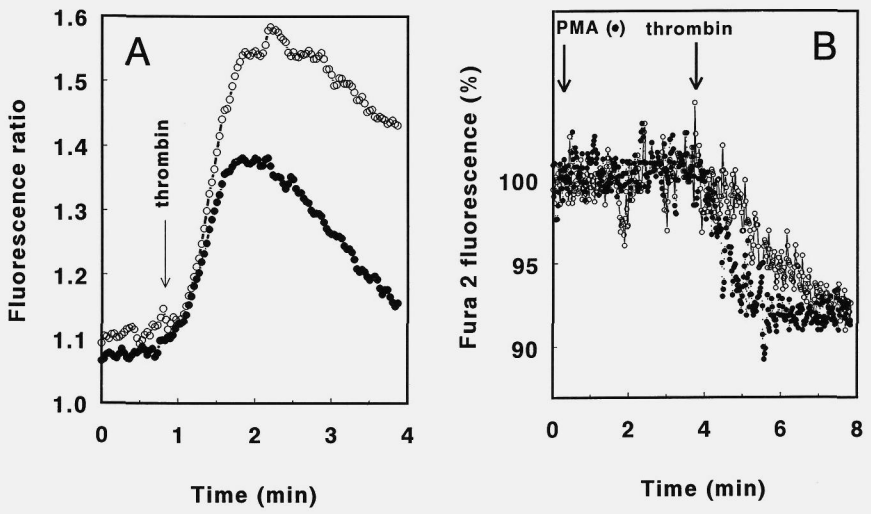

Figure 4: Cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$) and Ca^{2+} influx in fura 2-loaded HUVEC. A: the fluorescence ratio of 340/380 nm of the fura 2/calcium complex is shown (mean of three cultures) under basal conditions (○) and in the presence of PMA (10nM, ●). The addition of thrombin (1U/ml) is indicated. B: Representative experiment of Ca^{2+} influx determination. Fluorescence of fura 2 at its isobestic point (359 nm) is shown under basal condition (○) and in the presence of PMA (10nM, ●). The thrombin-induced Ca^{2+} influx was determined by quenching of fura-2 fluorescence by Mn^{2+}. Time points of addition of PMA and thrombin (with 1.6 mM Mn^{2+}) to the EC monolayers are indicated.

The thrombin-induced calcium ion influx was measured by determination of the rate of fura 2 fluorescence quenching (at 359 nm) by Mn^{2+}. The quench rate from the basal fluorescence to a lower steady state level was increased by a 5 min preincubation of HUVEC with PMA from 129 ± 15 s to 86 ± 10 s ($p<0.05$, 6 cultures; Figure 4B). Since the $[Ca^{2+}]_i$ decreases in the presence of PMA, in spite of the increase in calcium ion influx rate, this strongly suggests that the calcium-efflux is also enhanced by PMA.

Table 3: Effect of PMA (10 nM) on cyclic nucleotides contents in human umbilical vein EC.

	Cyclic nucleotide (pmol/3.5x10^5 cells)	
Addition	cGMP	cAMP
None	0.78 ± 0.14 (6)	2.25 ± 0.22 (6)
PMA	0.82 ± 0.13 (6)	2.17 ± 0.27 (6)
Thrombin	0.98 ± 0.12 (4)	2.06 ± 0.10 (4)
Thrombin+PMA	0.90 ± 0.06 (4)	2.11 ± 0.11 (4)
ANP	2.15 ± 0.55* (6)	2.40 ± 0.12 (6)
Forskolin	0.90 ± 0.14 (6)	9.98 ± 1.41* (6)

Endothelial cells were preincubated for 15 min with IBMX and then incubated with PMA for one hour in the absence or presence of thrombin (1 U/ml). ANP (10^{-9}M) and Forskolin (10 μM) were used as positive controls for cGMP and cAMP elevation, respectively. Data are expressed as mean ± SEM of the number of different cultures indicated in parentheses. *P < 0.05 vs control.

PMA does not alter the cytoplasmic cAMP, cGMP or ATP concentrations in HUVEC.

Previous studies have demonstrated that increases of cytoplasmic cAMP or cGMP concentrations are associated with a reduction in actin-non-muscle myosin interaction and a decrease in endothelial permeability.[3,14,29,38,53] The cyclic nucleotide concentrations were determined in HUVEC after incubation with 10 nM PMA and/or 1U/ml thrombin in the presence of the phosphodiesterase-inhibitor IBMX. PMA did not affect cytoplasmic cAMP or cGMP levels, both under basal- and thrombin-stimulated conditions (Table 3). Also in the absence of IBMX, incubation of the cells with PMA did not affect the cellular concentrations of cAMP and cGMP when determined 1, 2, 5, 10, 30 and 60 min after the addition of 10 nM PMA (cAMP level ranged from 85 to 109% of control and cGMP from 89 to 94% of control, 4 different cultures).

Inhibition of cyclooxygenase activity by 25 μM aspirin was without effect on the PMA-induced reduction of endothelial permeability (not shown), which excludes a possible indirect effect of PMA via prostglandin production.[7,12,16]

Because changes in the cellular ATP concentration also may affect endothelial contractility and permeability,[60] we evaluated the ATP level in HUVEC after exposure to PMA. The ATP level (5.4 $\pm$ 0.5 μM/3.5x10^5 cells, 3 different cultures) was not changed by 1, 10 or 100 nM PMA or 1 μM Ro31-8220 (being 6.2 $\pm$ 0.6 μM, 5.7 $\pm$ 0.8 μM, 6.4 $\pm$ 0.4 μM and 5.9 $\pm$ 0.3 μM, respectively, after a one hour incubation).

DISCUSSION

The present study shows that moderate activation of a calcium-dependent PKC reduces basal- and thrombin-stimulated permeability of human umbilical vein and microvascular EC monolayers. This effect is accompanied by a reduction of $[Ca^{2+}]_i$ and a disturbance of the actin-non-muscle myosin interaction, which both may contribute to the decrease in endothelial permeability. Our data on permeability agree with previous data of Yamada et al[62] on umbilical vein EC, but seem by contrast, to differ from those of Bussolino et al[5] and from studies on animal EC *in vitro*,[4,19,33,43,51] which used higher concentrations of PMA (100 nM or more) to stimulate the cells. At a high degree of PKC activation (100 nM) we also observed an increase in endothelial permeability. This suggests that different PKC may be involved in the regulation of endothelial permeability.

The increase of endothelial permeability induced by vasoactive substances and thrombin involves contraction of the marginal area of EC and formation of small intercellular gaps.[35] This is caused by interaction of actin and non-muscle myosin, which is primarily regulated by phosphorylation of the myosin light chains (MLC). Phosphorylation of the MLC is increased by Ca^{2+}/calmodulin-dependent activation of MLC kinase, and reduced by MLC phosphatases.[48,61] The PKC-induced reduction of permeability observed in our experiments points to a reduced actomyosin interaction. The colocalization of actin and non-muscle myosin was disrupted by incubation of PMA for one hour (Fig 3), which may indicate that the affinity of myosin for actin was diminished during PKC activation. Support for this possible mechanism is found in other cell types. Higashihara et al[21] reported that PKC phosphorylated MLC in platelets, and suggested that PKC inhibited the actin activated ATPase activity by decreasing the affinity of myosin for actin. However, Garcia et al[13] recently reported that PMA did not affect the phosphorylation of MLC in non-stimulated human and bovine EC. Additionally, it has been reported for gizzard stomach smooth muscle cells that phosphorylation of the MLC kinase by protein kinase C occurred at the same site

which was phosphorylated by cAMP-dependent protein kinase and reduced the activation of MLC kinase by Ca^{2+}-calmodulin.[8,25,40] However, whether this phosphorylation also occurs and causes a reduction of MLC phosphorylation in thrombin-stimulated human EC remains to be elucidated.

PKC activation probably regulates smooth muscle cell contraction.[2,24,45] There is no evidence that this is mediated by phosphorylation of MLC, though PKC may inhibit the MLC phosphatase activity.[30,36] Analogous to smooth muscle cells, the association of actin and non-muscle myosin in and contraction of EC may be promoted by PKC activation. A number of studies demonstrated that PKC activation changes the morphology of cells, including EC, and the appearance and localisation of F-actin in the cell periphery and stress fibres as well as that of F-actin associated proteins, such as vinculin and α-actinin in EC.[47,54,59] Proteins associated with F-actin, such as caldesmon, may regulate actomyosin interaction.[50,58] Endothelial caldesmon is phosphorylated upon PKC activation, which promotes the dissociation of caldesmon from F-actin and may facilitate actomyosin interaction.[52] Furthermore, while incubation of cells for 1 h with PMA causes the disappearance of the dense periferal actin bundles and an increase in centrally located stress fibres in porcine aorta and bovine corneal EC,[47,59] it caused major alterations in the distribution of actin in the cell periphery of human EC (this study;[5,55]), by which the cell margins obtained a wrinkled outline, but no stress fibres were formed. Additionally, in human EC the formation of thrombin-induced stress fibers and the simultaneously occurring association of non-muscle myosin therewith was entirely prevented by preincubation of the cells with PMA, similarly as observed after preincubation with the adenylate cyclase activator forskolin (Fig 3). On the other hand, Thurston et al[55] observed that inhibitors of PKC also caused a disappearance of thrombin-induced stress fibre formation. These observations suggest that PKC activation by PMA causes multiple effects on the EC cytoskeleton. This may in part depend on the types of PKCs available in the cell (see below).

Several mechanisms may be considered to play a role in the modulation of endothelial permeability by PKC activation. The $[Ca^{2+}]_i$ of thrombin-stimulated HUVEC was reduced when the cells were preincubated with PMA, though the calcium ion influx rate was elevated (Fig 4). This suggests that both influx and efflux of Ca^{2+} ions are accelerated by activation of PKC resulting in a reduced $[Ca^{2+}]_i$. These observations agree with previous reports. PMA reduced ATP- and thrombin-stimulated increase of $[Ca^{2+}]_i$ in umbilical vein EC,[7,16] although uptake of $^{45}Ca^{2+}$ was increased by PMA.[12] Furthermore, the thrombin-induced formation of inositol phosphates was inhibited by PMA in umbilical vein EC,[20] by which the Ca^{2+} release induced by inositol-(1,4,5)trisphosphate is reduced. Hence, reduction of $[Ca^{2+}]_i$ by PKC activation may be one mechanism contributing to the decrease in endothelial permeability induced by moderate PKC activation. PMA enhanced the activation of adenylate cyclase in

bovine EC.[31] It has also been reported that prostacyclin synthesis, which indirectly can enhance the cellular cAMP concentration,[1,23] was stimulated by PMA.[7] The effect was most pronounced after 2-3 hours PMA stimulation. This time course resembles the effect of PMA on the (thrombin-stimulated) permeability. However, we did not observe any effect of PMA on the cAMP concentration, nor could we block the PMA-induced reduction in permeability by the addition of the cyclooxygenase-inhibitor aspirin. This indicates that PKC and cAMP-dependent protein kinase act independently in reducing endothelial permeability. Elevation of the cytoplasmic cGMP concentration also induces relaxation of smooth muscle cells and EC.[3,14,44] Inhibition of cGMP formation by a reduction of the synthesis of the guanylate cyclase activator nitric oxide (NO) may induce the opposite effect. Inhibition of the constitutive NO synthase activity by PKC has been reported in bovine EC.[10,22] However we observed no significant effect of PKC activation by PMA on the cGMP concentration (Table 3).

A large number of different PKCs are recognized today. They comprise the Ca^{2+}-dependent PKCs (PKCα, PKCβ and PKCγ), the new PKCs (PKCδ, PKCϵ) and the atypical PKCζ, which does not respond to diacylglycerol.[41] Human umbilical vein EC possess α-, β-, ϵ- and ζ-subtypes of PKC, while the γ and δ-subtypes were not detectable.[5,37] The strongest reduction of endothelial permeability was obtained in our study by PMA (stimulates all PKCs), and by thymeleatoxin, which is relatively specific for calcium-dependent PKCs, i.e. PKCα and PKBβ in human umbilical vein EC.[46] On the other hand, activation of PKCβ1 by DOPPA, which induced E-selectin and VCAM-1 expression on umbilical vein EC (Deisher et al, 1993), did not reduce endothelial permeability. ß1-activation may be involved in the opposite effect, since the permeability increased at 100 nM DOPPA (Fig 2). Because 1-oleoyl-2-acetylglycerol (OAG) reduced thrombin-stimulated permeability, but does not affect PKCζ,[30,37] these results suggest that PKCα or PKCβ2 are involved in the reduction of endothelial permeability at a low or moderate degree of PKC activation. In addition, at higher levels PKC activators increase the endothelial permeability, suggesting that PKC activation has a dual effect.

Lynch et al[33] have suggested that thrombin-induced activation of PKC is necessary for the thrombin-induced increase in endothelial permeability. Although thrombin is a potent activator of PKC it is unlikely that 1 U/ml thrombin is a stronger PKC stimulator than 10 nM PMA in umbilical vein EC. Because such moderate levels of PKC activation result in a decrease in the permeability of HUVEC and human microvascular EC monolayers, the involvement of PKC activation in the regulation of thrombin-stimulated permeability is probably more complex involving both stimulatory and inhibitory elements. Our findings contrast to previous observations on EC monolayers of animal origin.[4,19,33,43,51] The exact reason for this disagreement is not yet known. Among the causes that may contribute to the

difference, three deserve special attention. The concentration of PMA in our experiments is lower than that in many other experiments. The basal permeability in our experiments is relatively low as compared to other experiments (5- to 10-fold lower). Finally, a difference in species may contribute. It is interesting to note that thrombin activates both phospholipase C and phospholipase D in umbilical vein EC.[15,16] Under our experimental conditions, the natural PKC activator diacylglycerol thus formed, like the diacylglycerol analogue OAG (Table 2), activates a feedback mechanism which limits the thrombin-induced EC contraction and permeability. However, the fact that PKC inhibitors Ro31-8220 and calphostin C did not significantly enhance thrombin-stimulated endothelial permeability in our experimental conditions may point to several mutually counteracting effects of PKC activation on endothelial permeability.

ACKNOWLEDGEMENTS

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CHAPTER 7

GENERAL DISCUSSION

7.1. Barrier function of endothelial cells in culture

The observation that intercellular gap formation is caused by endothelial cells (EC) pulling apart,[45] and the recognition that the endothelial actin-non-muscle myosin cytoskeleton is involved in this contraction,[41,63] suggested that endothelial permeability is similar but probably not identically regulated as the controlled actin-myosin contractile apparatus of smooth muscle cells. Knowledge about these cellular protein interactions and their regulation, may help us to predict how vascular leakage can be prevented. A valid *in vitro* model to study these mechanisms is of major importance. For that reason, great effort has been made to culture human EC to tight monolayers with *in vivo* features (see general introduction). Chapter 2 shows that endothelial monolayers from micro- or macrovessels have *in vitro* several features in common. Molecular sieving characteristics of micro- and macrovascular EC monolayers are similar, and agents that increase intracellular cAMP, like the adenylate cyclase activator forskolin and the ß-adrenergic stimulator isoproterenol, are equaly potent in reducing the permeability of micro- and macrovascular EC monolayers. cAMP-elevating agents that inhibit microvascular leakage *in vivo*[1,57,62] may thus directly act on the microvascular EC. These common features partly justify that we, and many other investigators, mainly use endothelial cells from large arteries and veins to study mechanisms of vascular leakage, which predominantly occurs *in vivo* in the microvasculature. On the other hand the presence of cGMP-dependent protein kinase (cGMP-PK) in foreskin microvascular EC and aorta EC, but not in umbilical vein EC (chapter 4), and the relatively active phosphodiesterase type III (PDE III) in umbilical vein EC compared to aorta EC, suggests that one can not speak about *the* continuous endothelium (see general introduction), but that different EC properties can be distinguished within continuous endothelium of various vascular beds and bloodvessels. The properties of EC are probably adapted to the local environment. Disappearence of the cGMP-PK during culture (chapter 5) emphasizes that freshly isolated or early passage EC are preferred to late passage cultures.

7.2. Intracellular signal transduction pathways involved in reduction of human endothelial permeability (Figure 1 and 2)

7.2.1. cGMP-dependent pathways

One of the major results of this thesis is the demonstration that elevation of intracellular cGMP is associated with a reduction in human endothelial permeability, particularly when the endothelial barrier is decreased under thrombin-stimulated conditions (chapters 3, 4 and 5). This finding was strengthened by the recognition of two cGMP-dependent pathways in EC. First, the presence of the cGMP-dependent protein kinase type I (cGMP-PK) and the reduction of $[Ca^{2+}]_i$ by the cGMP-PK activator 8-pCPT-cGMP in aorta and microvascular EC provides a pathway, wherein activation of cGMP-PK by cGMP reduces the $[Ca^{2+}]_i$. Second, the presence of cGMP-inhibited phosphodiesterase in umbilical vein EC, by which cGMP inhibits cAMP degradation. The interaction of actin and non-muscle myosin, which causes contraction of the marginal area of EC and formation of small intercellular gaps,[45,76] is primarily regulated by the phosphorylation of the myosin light chains (MLC). This process requires activation of MLC kinase by $[Ca^{2+}]_i$ and calmodulin.[61,76] An increase of cAMP in EC decreases MLC phosphorylation by modulation of the MLC kinase activity.[49] Modulation of the cAMP and Ca^{2+} ion concentration will thus affect endothelial permeability. It would be of interest whether cGMP-PK phosphorylates and/or inhibits the activity of the MLC kinase, in analogy with platelets and smooth muscle cells.[52]

The cGMP-generating particulate guanylate cyclase integrated in the atrial natriuretic peptide (ANP) receptor is present in EC (chapter 3). Several *in vivo* studies point to a protective effect of ANP on the pulmonary protein extravasation, while the opposing effect is observed in the systemic microvasculature.[29,30,75,80] Pharmacological ANP concentrations reduce central venous pressure and may thereby reducing pulmonary artery hydrostatic pressure,[80] thus reducing pulmonary leakage. However, different endothelial features in the vascular beds may also contribute to these opposing effects. Especially, the (preferential) expression and activity of various types of PDE in EC of different vascular beds may explain different responses of EC to elevation of cGMP. cGMP-stimulated PDE is found in bovine aorta EC (cGMP promotes cAMP hydrolysis),[35] whereas the cGMP-inhibited PDE (cGMP inhibits cAMP hydrolysis) is highly active in human umbilical vein EC, but not in aorta EC (chapter 4).

cGMP is formed by activation of the soluble guanylate cyclase by NO. This provides an endogenous negative feedback mechanism for thrombin-stimulated endothelial permeability, because elevation of $[Ca^{2+}]_i$ by thrombin, particularly by Ca^{2+} ion influx,[37] activates the constitutive endothelial NO synthase (eNOS). Enhancement of the thrombin-induced increase in permeability by the NO synthase inhibitor L-NAME is in agreement with

this hypothesis (chapter 4). The counterregulatory mechanism probably is functional in a limited part of the endothelium. eNOS immunoreactivity is predominantly found in EC of large vessels.[6,43] The positive NO synthase-staining in the majority of human umbilical vein EC, while umbilical artery EC were mainly negative, is notable.[12] Vasodilator-stimulated phosphoprotein (VASP) may be an important target for cGMP/NO, with regard to regulation of endothelial permeability, because of its location at the endothelial margins. The protein is phosphorylated when cGMP is elevated in EC (chapter 5). Endothelial NO release causes phosphorylation of VASP in platelets and endogenous NO will, as activator of cGMP-generation, also induce VASP-phosphorylation in EC. Additionally, intracellular alkalinization by the thrombin-induced Na^+/H^+ exchanger may sustain eNOS activity by induction of Ca^{2+} ion influx.[18,20]

7.2.2. cAMP and endothelial permeability

Several *in vitro* and *in vivo* studies demonstrated that elevation of intracellular cAMP improves the endothelial barrier function.[8,28,36,40,68,78] This also holds true for microvascular EC (chapter 2). The macromolecular permeability decreases and the transendothelial electrical resistance increases of human EC monolayers from foreskin microvessels, pulmonary artery, umbilical vein and artery EC, under basal and thrombin-stimulated conditions, by addition of the adenylate cyclase activator forskolin (chapter 2 and 3). Prevention of cAMP degradation by inhibition of phosphodiesterase III or IV activity by SKF94120 or Indolidan and Rolipram, respectively, effectively reduced thrombin-stimulated permeability of umbilical vein EC (chapter 4). We found similar effects for other agents that increase cAMP, such as adenosine, ADP and 8-Bromo-cAMP in umbilical vein, aorta, pulmonary artery and iliac vein EC (not shown). Agonists of the ß-adrenoceptor-coupled adenylate cyclase epinephrine, norepinephrine, isoproterenol and salbutamol all reduced the endothelial permeability (chapter 2).[22,40] Minnear and colleagues[48] suggested a direct effect of isoproterenol on the actin filaments in pulmonary artery EC. Probably, cAMP prevents intercellular leakage by reducing the endothelial contraction. An increase in cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$) and phosphorylation of myosin light chain (MLC) by the MLC-kinase are involved in endothelial contraction.[76] However, cAMP does not affect the (thrombin-induced) increase in $[Ca^{2+}]_i$,[15,24] but rather decreases the MLC-kinase activity,[49] which results in a reduced interaction between actin and myosin and inhibition of gap formation. Baluk and McDonald[3] demonstrated in rat postcapillary venules that the ß2-agonist formoterol reduced the substance P-induced intercellular gap formation, rather than the gap size. cAMP elevation in umbilical vein EC by the prostaglandin PG-E_1 and the cAMP-analogue Sp-5,6-DCl-cBiMPS caused phosporylation of VASP, a protein located at cell-cell contacts (chapter 5). VASP may transduce the cAMP elevation in a reduced contractile

response, but this has to be proven. In particular, the thrombin-induced increase in endothelial permeability may be affected by adrenergic desensitization. Ishii and colleagues[31] demonstrated a heterologues desensitization by the ß-adrenergic receptor kinase 2 (ßARK2) of the thrombin receptor in Xenopus oocytes. ßARK2 inactivates the receptor by phosphorylation of serine and threonine residues on the receptor's cytoplasmic tail.

7.2.3. Protein kinase C and thrombin receptor

Activation of PKC by PMA in human umbilical vein EC causes reduction of the endothelial permeability both under basal and thrombin-stimulated conditions. This observation may be explained by a PMA-induced dissociation of the contractile proteins actin and non-muscle myosin or by a reduction in the (thrombin-induced) intracellular Ca^{2+}-ion concentration (chapter 6), but it contrasts to the effect of PMA in non-human EC monolayers. The discrepancy may be the consequence of the usage of different species and/or different PMA concentrations. The limited contents of PKC subtypes in human umbilical vein EC (PKC α, β, ϵ and ζ)[46] and the different substrate specificity of the PKC subtypes may be another explanation.[34]

The thrombin receptor-activity is reduced by homologues and heterologues desensitization. Activated thrombin receptors by thrombin on megakaryoblastic cell lines were internalized and mostly degraded.[25] Recovery of the thrombin response after this homologues desensitization required thus newly synthesized thrombin receptors. Furthermore, PMA induced phosphorylation of the thrombin receptor in these cells and caused loss of responsiveness to thrombin, which was prevented by the PKC inhibitor staurosporine. Homologues desensitization was not affected by staurosporine.[5] These findings are consistent with ours, inwhich PMA reduced thrombin-induced increase in permeability, but PKC inhibitors could not prevent this increase (chapter 6). PMA inhibited thrombin-induced Ca^{2+} ion mobilization in platelets,[79] which was attended by phosphorylation of the guanine-nucleotide-binding protein $G_i\alpha$. Phosphorylation of the G protein was also observed after activation by thrombin and inhibited by staurosporine. It is proposed that this mechanism serves as a negative feedback. Probably these mechanisms are also found in endothelial cells and may explain the reduction of the thrombin-stimulated permeability by PMA.

Under basal conditions another process may be involved in the PMA-induced permeability reduction. Caveolae are noncoated cholesterol-rich plasmalemmal vesicles found on several cell types, including EC. Disruption of the caveolae from rat lung microvascular EC by the sterol-binding compound filipin reduced albumin and insulin transcellular transport.[60] Smart and colleagues[65] have demonstrated in an epithelial cell line that PMA prevented internalization of caveolae. PKC activation may thus effect macromolecular transport through endothelial cell monolayers, but this have to be established yet.

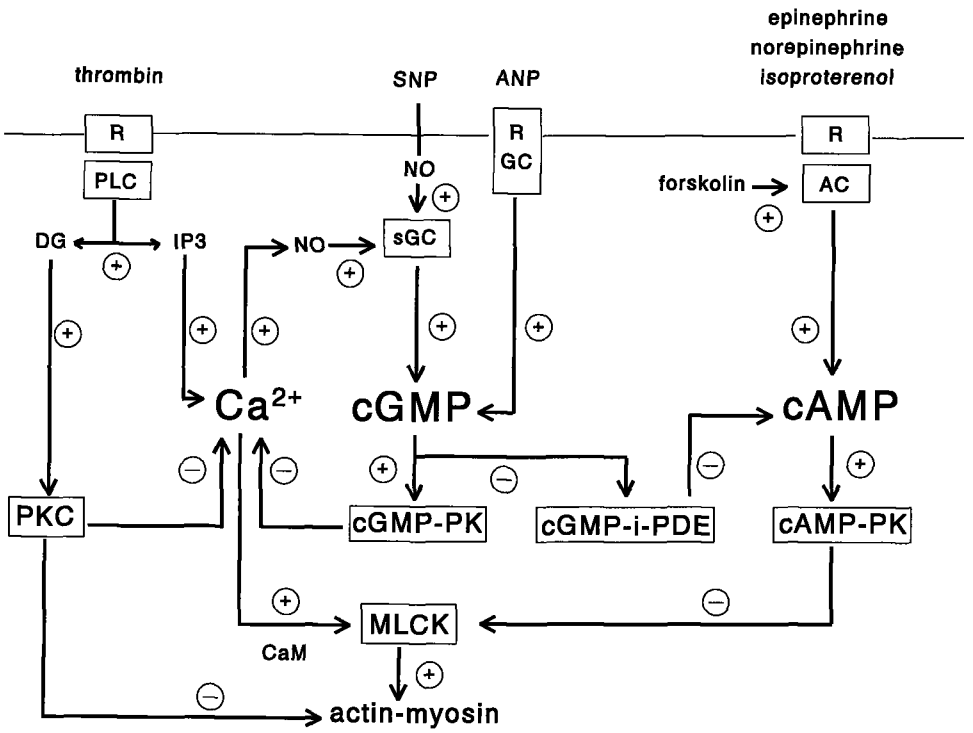

Figure 1: Intracellular signal transduction pathways recognized in human endothelial cells. Activation of PLC-coupled thrombin-receptors induces the formation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 promotes the release of calcium ions from intracellular pools. Ca^{2+} activates, together with calmodulin and ATP, the myosin light chain kinase (MLCK), which phosphorylates MLC. Phosphorylation of MLC leads to interaction of myosin and actin-filaments that provides the contracting force necessary to retract neighbouring EC from each other causing increased vascular permeability. This process is inhibited by guanosine 3',5'-cyclic monophosphate (cGMP), which is synthesized by activation of a particulate guanylate cyclase (GC) forming part of the atrial natriuretic peptide (ANP)-receptor, or by a soluble GC, which is activated by nitric oxide (NO). NO can be derived from an extracellular donor, such as sodium nitroprusside (SNP), or is formed by the Ca^{2+}-activated constitutive NO synthase, providing a negative feedback. cGMP activates the cGMP-protein kinase (cGMP-PK) in human aorta and foreskin microvascular EC. This enzyme may affect the intracellular calcium ion concentration ($[Ca^{2+}]_i$) in EC via a yet unknown mechanism. cGMP inhibits the cGMP-inhibitable-phosphodiesterase (cGMP-i-PDE) in human umbilical vein EC. The degradation of adenosine 3',5'-cyclic monophosphate (cAMP) by this type of PDE is thereby reduced. Intracellular cAMP levels can also increase by activation of ß-adrenoceptor-coupled adenylate cyclase (AC). The receptor complex is activated by epinephrine, norepinephrine and isoproterenol or AC can directly be activated by forskolin. cAMP reduces the MLCK activity probably via the cAMP-dependent protein kinase (cAMP-PK). Both cGMP and cAMP can thus, via distinct pathways, reduce or prevent endothelial contraction and permeability. PKC activation by DG may inhibit both an increase in the $[Ca^{2+}]_i$ and the interaction between actin and myosin. +:induction, -:inhibition

Phosphorylation of myosin light chain by MLC kinase is the initial event resulting in actin-myosin interaction and development of a contractile force in the endothelial cell.[76] This mechanism resembles that found in smooth muscle cells (SMC). In analogy, cAMP, cGMP, cAMP-dependent and cGMP-dependent protein kinase are all involved in relaxation of contracted SMC fibers.[11,19,53,54,56,66] Because of these similar characteristics and the fact that research of SMC contractility has been more developed than that of EC, it is worthwhile to follow the discoveries in this field. Interestingly, SMC tension is maintained during dephosphorylation of myosin ("latch state").[2] Furthermore, $[Ca^{2+}]_i$ plays a dual role: it dictates SMC myosin phosphorylation by MLC kinase, causing the initial force development, but inhibits this process by activation of CaM kinase II. This enzyme phosphorylates MLC kinase at high $[Ca^{2+}]_i$, which results in a reduced affinity of MLC kinase for Ca^{2+}/calmodulin and a decrease in the Ca^{2+} sensitivity of MLC phosphorylation.[72] MLC is also phosphorylated by PKC, but this does not activate contraction and is not involved in receptor-mediated MLC phosphorylation.[32,69] On the contrary, PKC-induced phosphorylation of MLC kinase can cause a decrease in the affinity of MLC kinase for calmodulin.[51] Despite these observations PKC activation seems to induce contraction via Ca^{2+}-dependent and independent pathways, which are largely obscure.[2,42,58]

7.3. Pathophysiological implications

To place our *in vitro* data in a physiological perspective, it should be realized that the leakage of fluid and macromolecules through the endothelial barrier is not only determined by the tightness of the latter, but also by the extent of capillary perfusion and by the vascular hydrostatic pressure (Starling forces), which is under control of the vascular tone. Agents that induce relaxation of endothelial cells, which improves endothelial barrier function, often cause relaxation of smooth muscle cells, simultaneously. The subsequent vasodilation decreases blood pressure in large vessels and arterioles, but can increase microvascular perfusion. As a consequence of these antagonistic actions of relaxation-inducing agents, different effects may be observed on the extravasation of fluid and macromolecules in various (micro)vascular beds. This holds true both for the effects of cAMP-elevating agents[71] and for those agents that increase the generation of NO and/or cGMP. In addition the NO/cGMP counter-regulatory mechanism may be more pronounced in endothelial cells from large vessels, because NO synthase activity was demonstrated mainly in large vessel endothelial cells by immunohistochemically technique.[6,43,77] The balance between effects of NO/cGMP-generating agents on barrier function and vasodilation may explain the different results found with various types of vascular beds.

Beneficial effects of NO/cGMP on the barrier function were observed in a number of experiments. Administration of the NO synthase inhibitor L-NAME to rats caused a

twofold increase in protein extravasation in left and right ventricle over that caused by noradrenaline, despite the fact that both agents evoked a similar increase in mean arterial blood pressure.[17] In feline small intestine, microvascular permeability increased after addition of L-NAME, which could not be explained by leukocyte-adherence or by an increase in microvascular pressure.[38] A protective role for NO in maintaining the microvascular integrity of the intestinal mucosa following acute endotoxin challenge was suggested by Hutcheson and colleagues.[27] The increased mortality rates of endotoxemic dogs treated with the NO synthase inhibitor N^{ω}-amino-L-arginine may also be caused by severe vascular leakage.[10] Inhalation of NO was also found to improve arterial oxygenation in patients with adult respiratory distress syndrome, a condition in which pulmonary hypertension is accompanied by vasoconstriction and pulmonary edema.[59] In the latter case, it has been suggested that increased arterial oxygenation was due to vasodilation. On the other hand, improvement of the endothelial barrier, which reduces the amount of extravasated fluid may additionally contribute to an increased gas exchange.

Vasodilation decreases blood pressure in large blood vessels and enforces here the improvement of the endothelial barrier by cyclic nucleotides. In these types of blood vessels, interference with NO production, as well as with cAMP or cGMP generation, would be expected to reduce the barrier function of the endothelium. If the permselectivity of the endothelial cells decreases, influx of large plasma constituents, such as LDL and β-VLDL, may increase, which may contribute to the development of atherosclerosis. This thougth is supported by the observation that in hypercholesterolemic rabbits neointima formation in the aorta is accelerated by chronic inhibition of NO production.[9]

In contrast to beneficial effects of NO on endothelial permeability, large amounts of NO have been reported to increase endothelial permeability. The number of venular leaky sites in the hamster cheek pouch after application of bradykinin or ADP was decreased by L-NAME and L-NMMA.[47] Substance P-induced edema in the rat skin was inhibited by both NO-synthase inhibitors.[26] High concentrations of NO may be toxic for endothelial cells and as such impair endothelial cell barrier function. In contrast to the effects on large vessels, predominance of arteriolar vasodilation may increase microvascular pressure exceeding the beneficial effect of endothelial cell relaxation.

Finally, continuous inhalation of NO by patients with pulmonary edema to promote oxygenation,[59] may have its limitations. Nitric oxide inhibits NO synthase activity, by reduction of the $[Ca^{2+}]_i$ or by binding to the heme group on NO synthase,[73] and thus may decrease endogenous NO production.[7] This could indicate that prolonged NO administration prevents benificial effects on pulmonary edema. Furthermore, the adventitia that surrounds the pulmonary vessels seems to be a specific barrier for NO. Administration of NO to the endothelial surface of rabbit pulmonary artery tissue relaxed the underlying SMC much more

effectively than NO from the adventitial site.[67] The role of endogenous NO on vascular permeability may depend on the experimental circumstances.[33,39] Chronical hypoxia upregulates the expression of NO synthase in the pulmonary vasculature, including the endothelium of small vessels.[77] The endogenous NO synthase in EC may therefore under hypoxic conditions be another target for a successful therapy.

Opposing effects on the vascular barrier, as noted for cGMP/NO, can also be made for cAMP. Agents, which elevated cAMP in and caused relaxation of smooth muscle cells more strongly than EC, increased blood flow and potentiated bradykinin-induced edema in rat skin. In contrast, edema was suppressed by agents, which mainly increased cAMP in EC.[74] Numerous beneficial effects of cAMP-increasing agents on the improvement of the microvascular barrier have been described. ß-adrenergic agonist and serotonin reduced petechiae on thrombocytopenic hamsters, probably by preventing the loss of microvascular integrity.[64] Histamine-induced edema in the hamster cheek pouch was succesfully reduced by ß2-adrenergic stimulation.[70] A protective role for ß-adrenergic agonists, acting on the microvascular EC, is expected in airway inflammation as in asthma and in systemic capillary leak syndrome.[14,16,55] Additionally, inflammatory mediators, such as histamine, stimulate sympathoadrenalmedullary discharge. These endogenous catecholamines inhibit microvascular leakage.[13,21,22] ß-adrenergic effects are probably elevated under ischemic conditions, because under these circumstances the externalization of ß-adrenoceptors is promoted.[44] Indeed, isoproterenol potently blocked ischemic-reperfusion induced capillary leakage in rabbit lungs.[1] This leakage was also prevented by inhibition of the cAMP phosphodiesterase and the cGMP-inhibited phosphodiesterase.[4,23] Phosphodiesterase inhibitors may reduce vascular leakage, because these inhibitors can reduce the pulmonary vascular tone by relaxation of smooth muscle cells, in addition to their effect on the endothelial barrier. Phosphodiesterase inhibitors are potential drugs for the prevention of vascular leakage. Probably, these drugs will not specifically affect the endothelium, because of the wide distribution of the various phosphodiesterase isotypes in different tissues.[50]

At the moment it is too early to speculate about the (benificial) effects of PKC activation on the vascular integrity. Because of the tumor-promoting activity of phorbol esters it is not obvious to activate PKC for improvement of the endothelial barrier function *in vivo*. However, the PKC subtypes may have different functions and be differently regulated by specific activators and inhibitors. Knowledge about the mechanism of PKC activation and inhibition may therefore in the future lead to the development of specific non-tumor PKC activators, which may be used for therapeutic purposes.

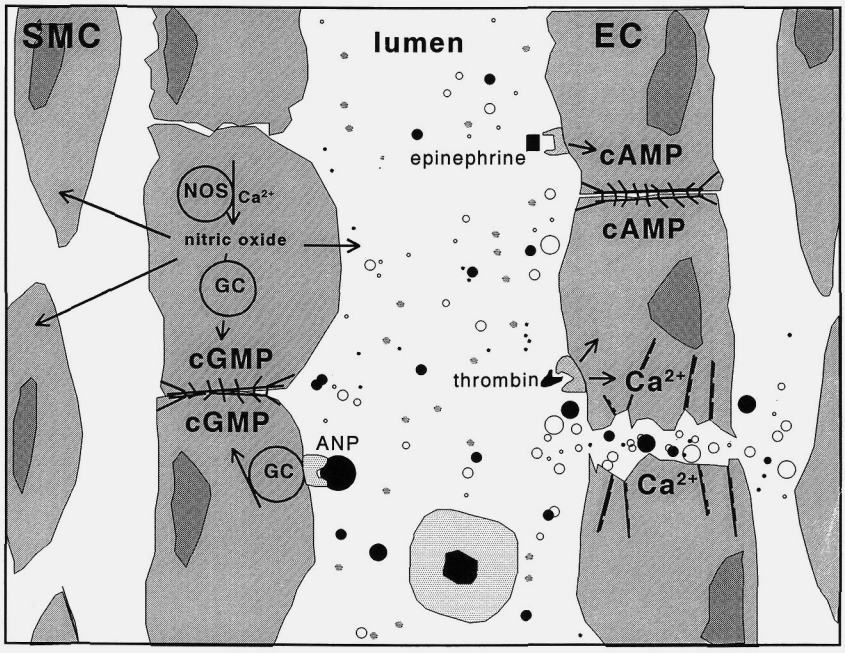

Figure 2: Effects of cGMP, cAMP and Ca^{2+} ions on human endothelial barrier function (see also figure 1). The endothelial barrier improves by agents, which induce the formation of cGMP and cAMP, such as ANP and epinephrine, respectively. In contrast, the release of Ca^{2+} ions, which is one of the consequences of thrombin-receptor activation, is associated with leakage of the endothelial cell monolayer. The Ca^{2+}-activated interaction of the contractile proteins F-actin and non-muscle myosin induces interendothelial gap formation. Endothelial cell-derived NO induces relaxation of SMC or inhibits platelet aggregation, but also activates the soluble guanylate cyclase in the endothelial cell. The NO-activated cGMP generation provides a counter-regulatory mechanism for a Ca^{2+} ion-involved increase in endothelial permeability, because at the same time the Ca^{2+}-dependent constitutive NO synthase is activated. EC, endothelial cell; SMC, smooth muscle cell; NO, nitric oxide; NOS, constitutive NO synthase; GC, guanylate cyclase; ANP, atrial natriuretic peptide.

7.4. Perspective

The findings decribed in this thesis provide a step forward in a better understanding of the intracellular mechanisms, which determine the endothelial barrier function, and give an indication for future work:

The effects of NO on cGMP in EC are demonstrated. Because cGMP/cGMP-PK activation causes phosphorylation of VASP, one would expect to observe phosphorylation of VASP when NO is generated during stimulation with thrombin, histamine or other compounds. This will extend the endogenous NO pathway to target proteins. Immunolocalization studies of NO synthase and cGMP-dependent protein kinase in human tissues will give information about the frequency of expression of these enzymes. It may help to predict the effect of certain cGMP-elevating agents on vascular leakage in a particular tissue. The same technique may demonstrate variation in expression of certain types of phosphodiesterases (as found in umbilical vein vs. aorta EC), which may give an indication for treatment of local vascular leakage by one particular phosphodiesterase-inhibitor. Furthermore, the disruption of actin-myosin colocalization by PMA has to be specified to which PKC subtype is responsible. More proof is needed for the loss of actin-myosin interaction, for instance by means of immunoprecipitation of actin/myosin and stabilization of actin by phalloidin. The discrepancy between PKC effects in human and non-human EC can be examined by simultaneous cultures of human umbilical vein or aorta and bovine aorta EC. In relation to pulmonary edema, cultures of human pulmonary microvascular EC are prefered above macrovascular EC. Our experience with non-contaminated foreskin microvascular EC cultures with barrier characteristics similar to aorta and umbilical vein EC cultures, suggests that human pulmonary microvascular EC cultures are available in the future. Regulation of endothelial permeability by cAMP-dependent protein kinase, cGMP-dependent protein kinase and PKC has been established. Therefore, several target proteins for these kinases may be considered, including MLC, MLC kinase, MLC phosphatase, VASP or other proteins at cell-cell contacts. Characterization of the features and regulation of these proteins will improve our knowledge about the intracellular mechanisms. The *in vitro* permeability model may approach the *in vivo* situation by several adaptations, including cocultures with smooth muscle cells and measurements under flow conditions or hydrostatic pressure. The relatively simple model is probably sufficient for most basal mechanistic questions.

The *in vitro* demonstration that vascular leakage by vasoactive substances is enhanced by NO synthase inhibitors, may be tested in an animal model by administration of a tracer molecule (ink, monastral blue), a stimulus (endothelin, PAF, bradykinin) and a NO inhibitor (L-NAME, L-NNA). Extravasation of the tracer molecule should be increased in the presence of the NO inhibitor. Finally, desensitization of EC to ß-adrenergic stimuli may be

verified in patients, which chronically inhale or orally take for instance salbutamol. An inflammation reaction induced by histamine in a skin test should in these patients, in case of desensitization, be less strongly inhibited by simultaneous application of salbutamol than in healthy volunteers.

7.5. REFERENCES

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SUMMARY

Inflammation or damage of endothelial cells causes vascular leakage and subsequently edema. In this thesis intracellular signal pathways were determined, which contribute to a reduction of vascular leakage (see Figure 1 of general discussion). The findings may guide the development of drug therapy for prevention of edema. The endothelial permeability of human endothelial cells of umbilical vein and artery, aorta, and foreskin microvessels, cultured to tight monolayers on porous filters, was used as an *in vitro* approach to vascular leakage.

Chapter 2 describes for the first time the molecular sieving characteristics of various types of human endothelial cells *in vitro*. The molecular sieving characteristics of microvascular endothelial cell monolayers are comparable to those found for macrovascular endothelial cell monolayers. The microvascular endothelial permeability is reduced by epinephrine, norepinephrine, isoproterenol and salbutamol via ß-adrenergic stimulation and is attended by elevated intracellular cAMP concentrations. A reduced response to ß-adrenergic stimulation appears during prolonged incubation of ß-adrenergic stimuli.

In chapter 3 data are shown that demonstrate that cGMP elevation in human umbilical artery and pulmonary artery endothelial cells correlates with a reduction in thrombin-induced increase in endothelial permeability. cGMP was in these cells increased by addition of atrial natriuretic peptide (ANP), the nitric oxide (NO) donor sodium nitroprusside (SNP) or the cell membrane-permeable analogue 8-Bromo-cGMP (8-Br-cGMP). An endogenous counter-regulatory mechanism via thrombin-activated (calcium-dependent) NO production was suggested by an increased permeability in the presence of the NO synthase inhibitor N^G-L-nitro-arginine methyl ester (L-NAME).

Two cGMP-dependent protein targets are described in chapter 4, that may mediate the effect of cGMP on endothelial permeability. The cGMP-dependent protein kinase activators 8-Br-cGMP and 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP) decreased thrombin-induced passage of macromolecules. Activation of cGMP-dependent protein kinase by 8-pCPT-cGMP decreased the accumulation of cytoplasmic calcium ions ($[Ca^{2+}]_i$) in human aorta endothelial cells. This demonstrated one cGMP-dependent mechanism, because elevation of $[Ca^{2+}]_i$ contributes to thrombin-induced increase in endothelial permeability, which was indicated by the intracellular calcium chelator BAPTA. The cGMP-inhibited-phosphodiesterase (PDE III), which degradates cyclic nucleotides, is a potential target for cGMP in human umbilical vein endothelial cells. The PDE III-inhibitors Indolidan and SKF94120 decreased considerably the thrombin-induced increase in permeability in these cells. The indication that NO can act *in*

vitro as an endogenous permeability-counteracting agent was strengthened by the observation, that the increase in endothelial permeability by L-NAME was prevented by simultaneous elevation of the endothelial cGMP concentration by atrial natriuretic peptide, sodium nitroprusside or 8-Br-cGMP.

Chapter 5 shows that various endothelial cell types contain cGMP-dependent protein kinase type I, including aorta, iliac vein and artery and foreskin microvascular endothelial cells, while umbilical vein endothelial cells lack this protein kinase. Stimulation with the cGMP-dependent protein kinase I activator 8-pCPT-cGMP inhibited the thrombin-stimulated permeability of monolayers of cGMP-dependent protein kinase I-positive EC, but not of umbilical vein endothelial cells. Similarly, 8-pCPT-cGMP largely prevented the thrombin-induced rise of $[Ca^{2+}]_i$, which was restricted to cGMP-dependent protein kinase I-positive EC. Activation of cGMP-dependent protein kinase I caused phosphorylation of vasodilator-stimulated phosphoprotein (VASP) localized at cell-cell contact sites. The involvement of VASP in endothelial permeability is not yet established.

Not only cAMP- and cGMP-dependent protein kinases, but also protein kinase C (PKC) seems to be involved in reduction of human endothelial permeability (chapter 6). The non-specific PKC activator PMA reduced both basal and thrombin-induced permeability of human umbilical vein endothelial cell monolayers. This effect was concentration-dependent and optimal at 10 nM PMA, but reversed at 0.1-1 μM. The involvement of a Ca^{2+}-dependent PKC (α or ß2) was suggested by PKC activators restricted to particular PKC subtypes. PMA decreased thrombin-induced calcium ion elevation, but did not affect intracellular cAMP or cGMP concentrations. Fluorescent-staining of the cytoskeleton suggested that PMA inhibits actin-non muscle myosin interaction, which may contribute to the effect of PMA on endothelial permeability.

These data indicate that agents, which increase cAMP or NO/cGMP in endothelial cells, improve the endothelial barrier function *in vitro*. Particularly, the NO-activated cGMP generation and pathways regulated by cGMP show qualitative and quantitative differences between endothelial cells from various vascular beds. This may determine the effectiveness of certain cGMP-elevating agents on the endothelial barrier function *in vivo*. Whether elevation of cAMP, NO and cGMP in the endothelium will have beneficial effects on the vascular barrier *in vivo*, depends on whether and how the hydrostatic pressure, in a particular blood vessel or vascular bed, will be affected simultaneously. The regulatory role of PKC activation on the endothelial barrier function is just recently recognized. The precise involvement of PKC has yet to be determined, but its activation seems to induce opposing effects on the endothelial permeability, rather than, solely an increase in permeability, as generally assumed.

NEDERLANDSE SAMENVATTING

Het endotheel bedekt de binnenzijde van alle bloedvaten. Ondanks dat het endotheel slechts één cellaag dik is, voorkomt deze barrière dat eiwitten, zouten en vloeistof uit de bloedbaan in het achterliggende weefsel treden. Onder omstandigheden dat het endotheel geaktiveerd (of beschadigd) is, kan de barrière functie ernstig verstoord zijn. Een verslechterde barrière kan onder andere leiden tot levensbedreigend long oedeem. Het is daarom noodzakelijk kennis te vergaren omtrent de in de endotheel cel (EC) optredende veranderingen tijdens EC aktivatie. Een beter begrip van de betrokken mechanismen zal uiteindelijk moeten leiden tot een effectievere behandeling van patiënten die lijden aan vasculaire lekkage. Het onderzoek dat in dit proefschrift beschreven staat heeft zich toegelegd op het vaststellen van mechanismen die betrokken zijn bij een verhoogde endotheel permeabiliteit als gevolg van een verminderde aansluiting tussen aangrenzende EC. Humane EC werden geïsoleerd uit navelstreng venen en arteriën, aorta's en long arteriën (macrovasculair) en uit bloedvaatjes van de voorhuid (microvasculair). Vervolgens werden de cellen op poreuse filters gekweekt tot een dichte cel monolaag, waarvan de barrière capaciteit bepaald werd door de doordringbaarheid van macromoleculen (dextranen, peroxidase, e.a.) te meten.

Ondanks dat vaatlekkage *in vivo* voornamelijk optreedt in (microvasculaire) postcapillaire venulen, wordt het onderzoek naar mechanismen die betrokken zijn bij vaatlekkage doorgaans uitgevoerd met gekweekte macrovasculaire EC. De reden hiervoor is dat microvasculaire EC minder eenvoudig te kweken zijn. Het is dan echter wel van belang vast te stellen in welke mate deze cel typen met elkaar overeen komen. Uit de resultaten beschreven in hoofdstuk 2 blijkt dat de moleculaire zeefkarakteristieken (moleculen met radii van 4.4 tot 162 Å en molecuul massa's van 180 tot 487000 Da) van EC monolagen afkomstig van humane voorhuid microvaten vergelijkbaar zijn met die van humane aorta en navelstreng venen. Aktivatie van de EC door het eiwit thrombine veroorzaakt een toename van de intracellulaire calcium-ion concentratie ($[Ca^{2+}]_i$), gepaard gaande met een contractie van de EC, wat leidt tot de vorming van intercellulaire openingen, verhoogde endotheel permeabiliteit en een verlaagde transendotheliale electrische weerstand. Dit treedt in elk EC type op (hoofdstuk 2 en 4). Thrombine-geïnduceerde toename van endotheel permeabiliteit is als model gebruikt voor de bestudering van vaatlekkage *in vitro*. Adrenerge stimulatie van microvasculaire EC met epinephrine en norepinephrine verminderde de basale en thrombine-geïnduceerde permeabiliteit, overeenkomstig de effecten op macrovasculaire EC. Aktivatie van ß2-adrenerge receptoren is hiervoor verantwoordelijk, gezien (1) de permeabiliteit afname door de specifieke ß-adrenerge agonisten isoproterenol (ß1 en ß2) en salbutamol (ß2), (2) de

remming hiervan door de ß-adrenerge antagonist propranolol en (3) de toename van de intracellulaire concentratie van adenosine 3',5'-cyclisch monofosfaat (cAMP; het aan ß-receptoren gekoppelde adenylaat cyclase genereert cAMP). Langdurige ß-adrenerge stimulatie (18 uur) leidt echter tot een desensitizering van de EC voor ß-adrenerge permeabiliteit verlaging.

In tegenstelling tot het algemeen geaccepteerde verband tussen endotheliale permeabiliteit verlaging en een concentratie toename van het intracellulair signaal molecuul cAMP, is er minder bekend over de effecten van een ander signaal molecuul, nl. guanosine 3',5'-cyclisch monofosfaat (cGMP). De effecten van cGMP op endotheel permeabiliteit en processen waar cGMP bij betrokken is, worden beschreven in de hoofdstukken 3, 4 en 5. De cGMP concentratie werd via verschillende wegen verhoogd in arteriële EC van navelstreng en long (hoofdstuk 3). Er werd gebruik gemaakt van atriaal natriuretisch peptide (ANP), dat van origine opgeslagen ligt in granules van hart spiercellen, van natrium nitroprusside (SNP), dat een stikstof oxide (NO) donor is, en van celmembraan-permeabele cGMP-analogen (8-Br-cGMP, 8-pCPT-cGMP). ANP bindt aan zgn. B-receptoren, die één geheel vormen met membraan-gebonden guanylaat cyclase, het enzym dat cGMP genereert. NO passeert eenvoudig de celmembraan en aktiveert de in het cytoplasma aanwezige guanylaat cyclase. Indien de afbraak van cGMP door zgn. fosfodiesterases (PDE) verhinderd werd met behulp van de PDE-remmer IBMX, induceerden zowel ANP als NO een 3-voudige toename van cGMP. Met name als de barrière functie verstoord was tijdens EC aktivatie, door toediening van thrombine, ging de cGMP verhoging gepaard met een vermindering van de permeabiliteit. Celmembraan-permeabele cGMP-analogen verminderden eveneens de permeabiliteit van (thrombine-gestimuleerde) EC monolagen afkomstig van aorta, navelstreng venen en microvaatbed.

De permeabiliteit toename door thrombine is mede het gevolg van een verhoging van de $[Ca^{2+}]_i$, daar het wegvangen van calcium-ionen door de calcium-chelator BAPTA de toename gedeeltelijk kon voorkomen. De specifieke cGMP-afhankelijke protein kinase (cGMP-PK) activator 8-pCPT-cGMP bleek, met name in aorta EC, de thrombine-geinduceerde $[Ca^{2+}]_i$-toename te kunnen remmen. In EC van navelstreng venen werd met specifieke remmers (SKF94120 en Indolidan) de aanwezigheid van fosfodiesterase III aangetoond. Dit enzym degradeert cyclische nucleotiden (cGMP, cAMP) en zijn aktiviteit wordt geremd door cGMP. SKF94120 en Indolidan verminderde de thrombine-geinduceerde toename in permeabiliteit en verhoogde de cAMP concentratie. Thrombine verhoogde in een groot aantal celkweken de cGMP concentratie. Dit kan verklaard worden door de aanwezigheid van de constitutive NO synthase in EC. Het enzym genereert NO en wordt geaktiveerd door calcium-ionen. NO aktiveert de cytoplasmatische guanylaat cyclase en kan op deze manier cGMP vorming induceren. NO (en cGMP) is mogelijk betrokken bij een

endogeen tegen-regulerend mechanisme wanneer de permeabiliteit verhoogd is: remming van NO produktie door de NO synthase remmer L-NAME versterkte de thrombine-geinduceerde permeabiliteit toename. ANP, SNP en 8-Br-cGMP verhinderden dit effect.

De cGMP-PK type I werd immunocytochemisch aangetoond in recent geïsoleerde en gekweekte EC van aorta, ilium arterie en vene en van het voorhuid microvaatbed. Het cGMP-PK substraat vasodilator-gestimuleerd fosfoprotein (VASP) werd in deze endotheel celtypen gefosforyleerd indien de cellen gestimuleerd werden met 8-pCPT-cGMP. Het belang van VASP in endotheel permeabiliteit is nog onduidelijk, maar de lokatie van het protein bij cel-cel contacten suggereert een mogelijke rol in de integriteit van de monolaag. cGMP-PK expressie en aktiviteit verdween gedurende langdurig kweken van de cellen en kon eveneens niet aangetoond worden in EC van navelstreng venen.

Naast een toename van $[Ca^{2+}]_i$, leidt stimulatie van EC door thrombine tot een aktivatie van protein kinase C. Verscheidene studies suggereren dat protein kinase C aktivatie van belang is bij de thrombine-geïnduceerde toename van endotheel permeabiliteit. De PKC aktivator PMA veroorzaakt in navelstreng vene EC echter geen verhoging van de permeabiliteit, in tegendeel PMA verlaagt de permeabiliteit (hoofdstuk 6), dat voorkómen kon worden door de cellen te preïncuberen met de PKC remmer Ro31-8220. Daarentegen konden de effecten van thrombine op de permeabiliteit niet verhinderd worden door Ro31-8220 en een andere PKC remmer calphostin C, terwijl PMA dit gedeeltelijk wel deed. De reductie door PMA van de thrombine-geïnduceerde toename in permeabiliteit werd eveneens gevonden met OAG (een diacyl glycerol analoog: een algemene PKC aktivator) en thymeleatoxin (een aktivator van calcium-afhankelijke PKC) in zowel EC van navelstreng venen, als van het microvasculair vaatbed. Stimulatie van EC met PMA bleek de thrombine-geïnduceerde $[Ca^{2+}]_i$-toename te verminderen, maar had geen effect op cAMP, cGMP of ATP niveau's in de EC. Interaktie van de contractiele eiwitten F-actine en myosine in EC kan leiden tot de vorming van intercellulaire ruimtes, en een toename in endotheel permeabiliteit veroorzaken. De thrombine-gestimuleerde colokalizatie van F-actine en myosine in de EC werd verstoord na toediening van PMA, wat dus mogelijk een verklaring geeft voor de gevonden effecten van PMA op endotheel permeabiliteit.

In conclusie: Verhogen van de intracellulaire cAMP, cGMP en/of NO concentratie en mogelijk de aktivatie van een (specifieke) protein kinase C bevordert het behoud of herstel van de humane endotheel barrière.

ABBREVIATIONS

ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular calcium ion concentration
cAMP	adenosine 3',5'-cyclic monophosphate
cAMP-PK	cAMP-dependent protein kinase
cGMP	guanosine 3',5'-cyclic monophosphate
8-Br-cGMP	8-bromo-cGMP
8-pCPT-cGMP	8-(-4-chlorophenylthio)cGMP
cGMP-PK I	cGMP-dependent protein kinase type I
DOPP	12-deoxyphorbol-13-phenylacetate
DOPPA	DOPP-20-acetate
EC	endothelial cell
FITC	fluorescein isothiocyanate
FSMVEC	foreskin microvascular endothelial cells
GC	guanylate cyclase
HAEC	human aorta endothelial cells
HRP	horseradish peroxidase
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cells
IBMX	isobutyl methylxanthine
kDa	kilo Dalton
L-NAME	N^G-nitro-L-arginine methyl ester
MLC	myosin light chain
NO	nitric oxide
OAG	1-oleoyl-2-acetyl-sn-glycerol
PC	permeability coefficient
PDE III	phosphodiesterase type III
PECAM-1	platelet/endothelial cell adhesion molecule 1
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
4α-PDD	4α-phorbol 12,13-didecanoate
SEM	standard error of the mean
SMC	smooth muscle cells
SNP	sodium nitroprusside
TEER	transendothelial electric resistance
Tx	thymeleatoxin
VASP	vasodilator-stimulated phosphoprotein

Curriculum Vitae

Richard Draijer werd op 17 december 1964 geboren te Zandvoort. In 1984 werd het diploma Atheneum behaald aan het Kennemer Lyceum te Overveen, waarna in hetzelfde jaar aangevangen werd met de studie Biologie aan de Rijksuniversiteit Leiden. Na gekozen te hebben voor de specialisatie richting Biochemie werden de laatste twee jaar van de studie besteed aan stages bij achtereenvolgens de vakgroep Celbiologie van de Rijksuniversiteit Leiden (prof. dr. P.J.M. van Haastert) en de vakgroep Immunologie van de Kinderkliniek van het Academisch Ziekenhuis Leiden (dr. M.J.D. van Tol). In 1990 werd de studie Biologie voltooid. In de maanden oktober 1990 - januari 1991 werd als wetenschappelijk medewerker bij het farmaceutisch bedrijf Centocor te Leiden elektronen microscopisch onderzoek verricht. Van februari 1991 tot februari 1995 was hij werkzaam als assistent in opleiding bij het Gaubius Laboratorium TNO Preventie en Gezondheid, sectie Pathophysiologie van het Endotheel te Leiden, onder leiding van dr. V.W.M. van Hinsbergh en prof. dr. P. Brakman. De resultaten van dit promotie onderzoek staan in deze dissertatie beschreven. Sinds mei 1995 is hij post-doc bij de sectie Lipiden (dr. H.M.G. Princen) van hetzelfde instituut.

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