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REVIEW

Molecular Mechanisms of Thrombin-Induced Endothelial Cell Permeability

N. V. Bogatcheva, J. G. N. Garcia, and A. D. Verin*

School of Medicine, Johns Hopkins University, Baltimore, MD, 21224 USA; E-mail: Averin@welch.jhu.edu

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Abstract—Confluent endothelium serves as a selective barrier between the vascular space of blood vessels and underlying tissues. Compromised barrier function of the endothelium in response to inflammation mediators, such as thrombin, is accompanied by reversible cell rounding and interendothelial gap formation. Endothelial barrier integrity substantially depends on the cytoskeleton, which ensures actin stress fiber formation and via actomyosin-driven contraction regulates cell shape and adhesion. Recent studies have shown the sequence of events that mediate signal transduction in endothelial cells. Binding of thrombin with its receptor initiates activation of heterotrimeric G-proteins, which, in turn, entails a decrease in cAMP level in the cell, increase in intracellular Ca^{2+} and diacylglycerol concentration, and activation of the small G-protein Rho. Phosphorylation of myosin light chains as a result of activation of myosin light chain kinase and inactivation of myosin phosphatases stimulates stress fiber formation and triggers actomyosin contraction. In addition, thrombin-induced rearrangement in the endothelial cytoskeleton is regulated by Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and tyrosine protein kinases. This review focuses on presently known biochemical mechanisms of cell response to thrombin and their role in endothelial barrier dysfunction.

Key words: thrombin, endothelium, barrier function

The endothelial cell (EC) layer inside blood vessels forms a semi-permeable dynamic barrier between the vascular space of blood vessels and underlying tissues. In normalcy, ECs tightly adhere to each other and weakly interact with the cells circulating in blood. An exception to this rule is endothelium of specialized vessels (e.g., lymph node venule endothelium). Inflammatory cytokines, viruses, and growth factors affecting EC properties can change endothelial barrier functions, thus disrupting the segregation between blood plasma and interstitial fluid. Among other physiological agents that cause endothelial dysfunction, thrombin is a special case. This protease produced on the surface of injured endothelium from prothrombin circulating in blood not only induces blood coagulation, but also exerts a specific effect on endothelium, causing disturbance of its barrier function and stimulating the release of inflammatory mediators, vasoregulatory agents, and growth factors. In addition, thrombin induces leukocyte adhesion on the EC surface and their subsequent penetration into the underlying tissues. Most effects of thrombin on endothelium are mediated by thrombin receptor and require the proteolytic activity of thrombin.

THROMBIN AND ITS RECEPTOR

Thrombin is produced by proteolytic cleavage of prothrombin in the so-called "prothrombinase complex". Factors V, X, calcium, and membrane phospholipids are required for proteolytic cleavage [1]. Human thrombin is a complex formed by the A chain (49 amino acid residues) and catalytic B chain (259 amino acid residues) connected through a disulfide bond. It was shown that further cleavage of 13 amino acid residues from the A chain can occur [1]. The thrombin globule forms a so-called anion-binding exosite on its surface, which participates in thrombin binding with substrates [1].

The 47-kD thrombin receptor (proteinase-activated receptor, PAR) is similar in its structure to G-protein-

Abbreviations: CAMK) Ca^{2+}/calmodulin-dependent protein kinase; EC) endothelial cells; FAK) focal adhesion kinase; IP_3) inositol-1,4,5-trisphosphate; MAPK) mitogen-activated protein kinase; MLC) myosin light chains; MLCK) myosin light chain kinase; MYPT) myosin phosphatase regulatory subunit; PAR) proteinase-activated receptor; PKA) cAMP-dependent protein kinase (protein kinase A); PKC) protein kinase C; ROK) Rho-dependent protein kinase; VASP) vasodilator-stimulated phosphoprotein.

* To whom correspondence should be addressed.

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coupled receptors containing seven transmembrane domains [2]. PAR is inactivated in a unique way: interaction with thrombin results in proteolysis of the 41-amino-acid residue N-terminal exodomain. The new amino acid sequence formed serves as a bound ligand itself [2]. Four types of PARs are currently known, of which three are thrombin-activated and one is trypsin-activated [2-5]. In EC, thrombin-activated PAR-1 and trypsin-activated PAR-2 was found [6]. It is believed that the later is activated *in vivo* by tryptases secreted by mastocytes [6]. It was shown that some effects of thrombin, such as increase in the capillary permeability coefficient, increase in pulmonary artery pressure, and increase in the pulmonary weight due to developing edema, are completely absent in mice deficient in the PAR-1 gene [7].

Synthetic peptides containing the SFLLRN sequence downstream to the C-terminus from the cleavage site in human PAR-1 can activate PAR-1 in the absence of thrombin, although much less effectively [8, 9]. These peptides perhaps bind to PAR-1 less tightly, or the large thrombin molecule serves as an additional ligand for PAR-1, enabling conformational changes in PAR that cause activation. The efficiency of thrombin binding to PAR-1 is apparently ensured by the interaction between the anion-binding exosite of thrombin and the receptor sequence located downstream to the C-terminus from the SFLLRN sequence [1]. In addition, antibodies raised against the proximal region of PAR-1 exodomain (amino acid residues 70-99) and the second extracellular loop of PAR-1 (amino acid residues 244-265) prevent thrombin-mediated activation of the receptor, which is indicative of multiple binding sites for thrombin on PAR-1 [10].

The fact that receptor activation cannot be reversed by dissociation of the agonist from PAR-1 implies that activation of PAR-1-containing cells should not depend on thrombin concentration, which is not true. It was shown that thrombin-induced EC activation, similar to activation of *Xenopus* oocytes or platelets, is dependent on thrombin concentration [2, 9, 11, 12]. For example, permeability of umbilical vein EC changes almost linearly at 0.001-1 μM thrombin or 10-1000 μM PAR-1 peptide agonist [9]. This phenomenon may be accounted for by the assumption that the interaction with the agonist causes temporal activation of the receptor, which is then inactivated by a specific mechanism. In EC, as in other cells, PAR-1 activation by thrombin or peptide results in desensitization of PAR-1 to both agonists [13, 14]. Restoration of cell sensitivity occurs within 90 min in the case of peptide stimulation and during a longer period if the cells are stimulated with thrombin [15]. Inhibitors of endo-/exocytosis and serine/threonine phosphatases but not protein synthesis inhibitors can delay resensitization of PAR-1 to the agonists at this stage [16, 17]. The second stage of resensitization lasts approximately 20 h and results in the occurrence of sensitivity both to the activating peptide and thrombin. This stage may be delayed by protein synthesis inhibitors [14, 16].

Thus, PAR-1 functions are regulated via its phosphorylation and internalization as well as degradation. Even 2 min after the addition of agonist to megakaryoblastic cells, 90% of PAR-1 is sequestered into endosomes via coated pits [16]. This occurs in response to the agonist peptide and thrombin, which demonstrates that internalization does not require the proteolysis of the receptor. It is known that thrombin-induced internalization of PAR-1 is regulated by phosphorylation of its cytoplasmic C-terminal domain [18]. PAR-1 is phosphorylated in *Xenopus* oocytes presumably by G-protein-coupled receptor protein kinase 3 [19]. A novel 33-kD protein kinase, which phosphorylates PAR-1 after stimulation with thrombin, has been found recently in platelets [20]. It remains unclear, however, which protein kinase is responsible for thrombin-induced phosphorylation of PAR-1 in EC. It was shown that protein kinase C capable of PAR-1 phosphorylation does not participate in the thrombin-induced PAR-1 phosphorylation [21]. The authors of [22] showed that umbilical vein ECs contain a substantial intracellular PAR-1 pool. Thrombin-stimulated PAR-1 is translocated from the Golgi organelle to the membrane surface. This mechanism may account for the relatively rapid recovery of response to thrombin in EC compared to other cells. It was recently discovered that the Hsp90 chaperone can interact with PAR-1 [23]. However, this interaction is likely to mediate signal transduction by a certain route rather than to determine the stability or ability of receptor activation on the whole [23].

PAR-1 is actively expressed in atherosclerotic plaques, whereas intact artery walls contain a rather small amount of this protein [24]. Conversely, PAR-1 is actively expressed in capillaries and venules [25]. For instance, umbilical vein EC contain approximately 10^6 PAR-1 molecules per cell [26]. It is PAR-1 that mediates the majority of thrombin effects on endothelium. For example, barrier dysfunction, prostaglandin synthesis, and expression of specific growth factors may be induced by PAR-1-activating peptides [27]. However, it was shown that proteolytically inactive thrombin can change the permeability of the endothelial monolayer [28] and that thrombin tetradecapeptide, which also does not exert proteolytic activity, induces EC proliferation [29]. The mechanism of this process remains obscure. It was shown that thrombin contains a chemotactic site and an EC linkage site (Arg-Gly-Asp). The interaction of the cells with these thrombin sites does not require proteolytic activity of thrombin and involves binding sites of the cell surface that are most likely different from PAR-1 [25].

G-PROTEINS AND G-PROTEIN-TRIGGERED CASCADES

Guanine nucleotide-binding regulatory G-proteins mediate the interaction between PAR-1 and cytoplasmic or membrane effectors, such as phospholipases and ion channels. Heterotrimeric G-proteins have a characteristic structure where the α-subunit contains a nucleotide-binding site and, in some cases, a site for ADP-ribosylation by a bacterial toxin. The β- and γ-subunits form isoprenylated heterodimer tethering the whole complex to the membrane. Receptor activation resulting in the interaction of the receptor with G-protein α-subunit triggers the exchange of bound GDP for GTP, thereby the heterotrimer dissociates, and either α-subunit or βγ-heterodimer or both interact with the target protein [30, 31]. It was shown that thrombin receptor can interact with several different G-proteins, thus triggering multiple signal mechanisms. It can be assumed that the set of G-proteins with which PAR-1 interacts in certain cells and tissues as well as isomorphous composition and sensitivity to G-proteins of effector proteins will determine the pattern of tissue-specific physiological response to thrombin. In EC, similar to many other cells, PAR-1 is linked to the pertussis toxin-sensitive G_i, whose activation is coupled with adenylate cyclase inhibition [32]. In addition, PAR-1 interacts with G_q, which activates phosphoinositide-specific phospholipase C [33]. PAR-1 is also linked to $G_{12/13}$ [33], which is responsible for the activation of the small G-protein Rho [34].

G_i-dependent adenylate cyclase inactivation results in a decrease in the intracellular cAMP concentration and inhibition of cAMP-dependent protein kinase (protein kinase A, PKA). The main consequences of this process will be considered below.

G_q-dependent activation of phospholipase C (presumably its β isoform [1]) accelerates phosphatidylinositol 4,5-bisphosphate cleavage to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. This, in turn, leads to an increase in the intracellular Ca^{2+} concentration and activation of protein kinase C (PKC). The activity of phosphoinositide-specific phospholipase C is regulated in many cells not only by G_q α-subunits, but also by G_i βγ-subunits [1]. In addition, β isoform of phospholipase C can undergo phosphorylation by PKA [35], and PKA inhibition as a result of G_i activation may increase phospholipase C sensitivity to G_q. However, in EC thrombin-induced increase in Ca^{2+} and IP_3 concentration is not inhibited by pertussis toxin [36], which is indicative of a crucial role of G_q-proteins in phospholipase C activation in EC.

Activation of the small G-protein Rho is mediated by the guanine nucleotide exchange factor, which, in turn, can be regulated not only by $G_{12/13}$ α-subunit, but also by G_q α-subunit and G_i α or βγ-subunits [37]. However, the expression of constitutively active G_{12} and G_{13} but not G_{i2} or G_q α-subunits is sufficient for Rho-mediated induction of stress fiber and focal adhesion plaque formation in fibroblasts [34]. Thus, $G_{12/13}$/Rho-regulated rearrangement of the cytoskeleton may occur in nonmuscle irrespective of $G_{i/q}$, adenylate cyclase inhibition, phospholipase C activation, Ca^{2+} mobilization, and protein kinase C activation. Rho effector proteins in the cell are Rho-associated protein kinase (ROK) and the p140mDia protein [38]. It is believed that ROK regulates binding of integrins with the extracellular matrix, whereas p140mDia changes the local configuration of profilin, which modulates actin polymerization [38]. Other mechanisms whereby Rho participates in the thrombin-induced increase in the endothelium monolayer permeability will be discussed below.

Similar to many other cells, in endothelium Ca^{2+} regulates the activity of myosin light chain kinase (MLCK), Ca^{2+}/calmodulin-dependent kinase (CAMK), and calcium-dependent PKC isoforms. In addition, Ca^{2+}/calmodulin reverses inhibition of actomyosin ATPase by caldesmon [39, 40]. PKC activation is accompanied by suppression of phospholipase C activity and decrease in Ca^{2+} concentration [41]. This apparently ensures a rapid block of the thrombin-induced cascade.

Thrombin induces activation of tyrosine protein kinases Src, FAK, JAK, and Syk [1]. PKC presumably regulates Src activation [1], whereas MLCK is involved in the regulation of Syk activity in platelets [42]. Tyrosine kinases are involved in the regulation of Ras activity by G_i βγ with subsequent activation of Raf, which induces mitogen-activated protein kinases (MAPK) [1]. In addition, an elevated level of cAMP in the cells inhibits Raf activity. It is believed that thrombin regulates Raf activity not only via Ras activation, but also by decreasing cAMP concentration [1].

The activity of phospholipases A_2 and D also increases in thrombin-stimulated EC [41, 43, 44]. Presumably, $G\alpha_{i2}$, Ca^{2+}, PKC, and MAPK are involved in phospholipase A_2 activation, whereas phospholipase D is activated by PKC, tyrosine kinases, and Rho [1, 37, 41]. Thrombin-induced activation of phosphoinositide 3-kinase is mediated by tyrosine protein kinases, Rho, and, possibly, Raf [1].

Although more and more data on the participation of Rho, MLCK, and PKC in thrombin-induced disruption of EC monolayer integrity are accumulating, the role of G_i, MAPK, tyrosine protein kinase Src, and phosphoinositide 3-kinase in thrombin-dependent changes in the cytoskeleton remains discrepant [45]. A possible role of the Rho-related G-protein Rac in the thrombin-induced changes in the cell shape is also discussed [45].

MECHANISMS OF REGULATION OF ENDOTHELIUM PERMEABILITY

The data accumulated to date suggest agonist-induced increase in the endothelium monolayer perme-

ability. The monolayer integrity is regulated by the equilibrium between the forces that induce EC contraction and those that hold EC in the flat state. Activation of the former and suppression of the latter entail the occurrence of gaps between the cells and an increase in transendothelial permeability [39, 40, 46]. Stress fiber formation and activation of actomyosin interaction make the main contribution to the contractile response of the cells. Passive cell retraction as a result of cytoskeleton rearrangement and attenuation of cell–cell and cell–substrate junctions also plays a key role in EC contractile response [39, 40, 46]. For instance, the results of measuring the isometric contraction of umbilical vein EC grown on collagen substrate and comparing these data with the changes in the monolayer electric resistance suggest that contraction is responsible for the rapid and short-term (for several minutes) response of EC to thrombin. Attenuation of the junctions apparently contributes to both the rapid and slow (more than 60 min) stages of EC response [11]. According to other data, the isometric contraction of umbilical vein EC may continue up to 60 min [47]. The F-actin-disrupting agent cytochalasin D completely inhibits tension development in the stimulated EC [48]. Conversely, the F-actin-stabilizing agent phallacidin decreases the thrombin-induced increase in monolayer permeability [49], apparently by blocking cytoskeleton reorganization during which the peripheral F-actin disappears and multiple stress fibers piercing the EC center are formed.

ROLE OF INOSITOL-1,4,5-TRISPHOSPHATE AND CALCIUM IN BARRIER DYSFUNCTION

It is known that IP_3 triggers Ca^{2+} release from intracellular stores. IP_3 concentration in pulmonary artery EC increases approximately twofold during the first 10 sec after stimulation with 0.1 μM thrombin and then immediately decreases [50]. In umbilical vein EC, thrombin causes a 3.5- to 5-fold increase in IP_3 concentration [51, 52]. In aorta EC, however, no increase in IP_3 concentration was detected [51].

The fact that cytoplasmic Ca^{2+} regulates EC barrier function has been confirmed many times. It was shown that calcium ionophores increase endothelial permeability [53] and that the thrombin effect is attenuated under calcium shortage [54]. It was also shown that thrombin α increases Ca^{2+} concentration in EC by mobilizing Ca^{2+} from the intracellular stores and via its influx into the cell [55]. The first process is responsible for the rapid and short-term increase in Ca^{2+} concentration, whereas the second causes a delayed but long-term increase [54, 55]. Calcium concentration in pulmonary artery EC sharply increases from 50 to 600 nM during 20 sec after thrombin addition and then immediately starts to drop [50, 53]. In umbilical vein EC, Ca^{2+} concentration increases only up to 250 nM, but it decreases much more slowly [56]. This finding additionally confirms the finding that physiologic response to thrombin depends on EC nature and may differ in its duration and extent.

PHOSPHORYLATION OF MYOSIN LIGHT CHAINS AND ITS ROLE IN THE REGULATION OF BARRIER FUNCTION

Similar to many other nonmuscle cells, contraction triggering in EC is regulated by phosphorylation of the myosin regulatory light chains (MLC). It was shown that phosphate incorporation into Ser19 and Thr18 of the regulatory MLC not only increases actomyosin ATPase activity, but also shifts the equilibrium between the folded and unfolded myosin forms [57], thus providing the assembling and functioning of the contractile apparatus of the cells. In pulmonary artery and umbilical vein EC, MLC phosphorylation is maximal 2 min after thrombin treatment. The degree of phosphorylation decreases by 5 min but does not reach the initial value even 15 min after stimulation by thrombin [53, 58, 59]. MLC phosphorylation is accompanied by a 60% increase in the F-actin content 90 sec after thrombin treatment; as a result, 90% of soluble myosin becomes associated with F-actin [47].

The major protein kinase that is responsible for MLC phosphorylation in EC is so-called nonmuscle myosin light chain kinase. In EC this enzyme is present in five isoforms [60]. It was shown that in smooth muscles the nonmuscle MLCK is expressed at relatively low level, being present together with a shorter smooth muscle isoform, whereas only the nonmuscle MLCK can be detected in EC [61]. The nonmuscle and smooth muscle MLCK are the products of the same gene. The longer nonmuscle MLCK contains a unique N-terminal domain comprising potential sites of phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and type II casein kinase [58]. In addition, at least one of the nonmuscle MLCK isoforms can undergo phosphorylation of tyrosine catalyzed by Src kinase [62]. In pulmonary artery EC, MLCK activity increases approximately twofold 2 min after thrombin stimulation and then rapidly drops [58], in complete accordance with Ca^{2+} concentration curve [53].

It is known that the effector of the small G-protein Rho, ROK, can phosphorylate MLC at the same sites as MLCK [63]. More and more data indicate that ROK together with MLCK regulates the contractile apparatus assembly in nonmuscle cells. For instance, inhibition of ROK results in the disappearance of the central stress fibers in fibroblasts, whereas inhibition of MLCK entails the disappearance of the membrane layer actin fibers [64, 65]. It is still unclear whether ROK along with MLCK directly phosphorylates MLC in response to thrombin

stimulation. However, it was shown that MLCK inhibition leads to a complete suppression of isometric tension development in EC [11].

Serine/threonine phosphatases, which are responsible for protein dephosphorylation in the cell, with respect to their substrate specificity and resistance to endogenous inhibitors and different toxins may be divided into type 1 and 2 phosphatases. The catalytic subunits of phosphatases 1 and 2a, which are most widely represented in the cell, are very similar [66]. It was shown that inhibition of phosphatase 2a does not affect the extent of MLC phosphorylation in EC and the endothelial monolayer permeability [67]. Conversely, inhibition of phosphatase 1 increases the extent of MLC phosphorylation in EC and results in cytoskeleton rearrangement and changes in endothelial barrier function in the absence of agonists [67, 68]. Only phosphatase 1, but not phosphatase 2a, was found in so-called myosin-enriched EC fraction [67]. These data are in good agreement with the current view that it is phosphatase 1 that is responsible for myosin dephosphorylation in the cell [66]. The activity of myosin-associated phosphatases in EC decreases 2- to 3-fold in 1 min after thrombin stimulation, is restored to the initial level by in 5 min, and is increased by 15 min [59]. Such mode of phosphatase response may account for the delay in MLC dephosphorylation after the rapid inactivation of MLCK in thrombin-stimulated EC.

In smooth muscles, myosin-associated phosphatase 1 is a heterotrimer comprised of: 1) catalytic subunit, which does not exhibit a high substrate specificity; 2) regulatory subunit (myosin phosphatase targeting subunit, MYPT), which is responsible for binding with myosin; and 3) small subunit, whose function is unknown [66]. Both in smooth muscles and endothelium myosin dephosphorylation is catalyzed by the phosphatase 1 catalytic subunit δ [67, 69, 70]. In EC, the latter forms a complex with 110- and 70-kD proteins. Proteins with a lower molecular weight corresponding to that of myosin phosphatase small subunit were not found [70]. The 110-kD protein was identified as MYPT1 (Verin and Garcia, unpublished data). The 70-kD protein apparently corresponds to one of the recently characterized novel regulatory subunits of MYPT3 [71] or p85 [72]. Interestingly, in contrast to MYPT1/2, MYPT3 decreases phosphatase catalytic subunit activity towards myosin [71], whereas p85, conversely, acts similar to MYPT1/2 [72]. The activity of MYPT1/2-containing enzyme towards myosin is significantly inhibited on phosphorylation of its regulatory subunit by ROK [66]. It was found that p85 also contains phosphorylation sites for ROK [72], whereas the shorter MYPT3 most likely lacks them [71]. Anyway, the presence of several regulatory subunits ensures that there is an additional mechanism of regulation of myosin phosphatase activity in the cell.

The inactivation of Rho by C_3-transferase from *Clostridium botulinum* completely inhibits the thrombin-induced increase in umbilical vein EC permeability [59] and increase in MLC phosphorylation [73]. Similar results were obtained in experiments with ROK inhibitor [74], which is indicative of the key role of ROK in the Rho-mediated barrier dysfunction and MLC phosphorylation in response to thrombin. The inhibition of myosin phosphatase restores the effect of thrombin on C_3-transferase-treated umbilical vein EC [59].

In pulmonary aorta EC, however, the inhibition of phosphatases 1 and 2b contributes to thrombin-induced barrier dysfunction and affects the extent of MLC phosphorylation [75]. It has been shown earlier that phosphatase 2b can dephosphorylate mollusk MLC [76]. It was found that in pulmonary artery EC the activity of this Ca^{2+}-dependent phosphatase is maximal 20 min after thrombin stimulation. The enzyme first undergoes dephosphorylation (2 min after stimulation), then (in 10-60 min) the extent of its phosphorylation significantly increases [75]. The protein kinase responsible for phosphatase 2b phosphorylation has not been identified yet. However, the aforementioned data suggest that, along with the changes in Ca^{2+} concentration, phosphorylation may regulate phosphatase 2b functions in EC.

Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE (CAMK) AND ITS ROLE IN BARRIER DYSFUNCTION

Thrombin induces CAMK activation and autophosphorylation. The activity of this enzyme reaches maximal value by approximately 5 min of thrombin stimulation [77]. CAMK inhibition significantly decreases the thrombin-induced increase in the monolayer permeability for ions and proteins [77]. CAMK can phosphorylate MLCK in smooth muscles, decreasing MLCK affinity for calmodulin and thus ensuring MLCK desensitization towards Ca^{2+} [78]. MLCK is phosphorylated in EC in the course of thrombin stimulation [77]. However, similar to MLC phosphorylation, this process is independent of CAMK activation [77]. Thus, CAMK regulates endothelial barrier dysfunction in a way that is different from MLCK phosphorylation.

We have shown that filamin, an actin-binding G-protein responsible for three-dimensional actin network formation, is phosphorylated by CAMK in thrombin-stimulated cells and then translocated to the membrane layer area [77]. An introduction into EC of myristilated filamin peptides containing a phosphorylation site for CAMK not only inhibits filamin phosphorylation and translocation, but also significantly decreases the change in the electric resistance of the thrombin-stimulated monolayer [77].

Further study of the role of CAMK may reveal other mechanisms whereby this enzyme regulates endothelial barrier dysfunction.

ROLE OF PROTEIN KINASE C IN THE REGULATION OF ENDOTHELIAL BARRIER FUNCTION

Thrombin activates PKC both in the membrane and cytosolic EC fractions [79]. PKC activity in both fractions increases after 5 min of thrombin stimulation, reaching a maximum by 15 min, and then after 60 min of stimulation returns to the control level [79]. Inhibition or proteolytic degradation of PKC attenuates thrombin-induced endothelial barrier dysfunction [80, 81], inhibiting MLC phosphorylation [53]. It was shown that *in vitro* PKC can phosphorylate MYPT1, decreasing phosphatase 1 activity towards myosin [82]. It is also known that myosin phosphatase inhibitor CPI-17 can undergo phosphorylation by PKC, which enhances its inhibitory properties [83]. CPI-17 is expressed in EC (Verin and Garcia, unpublished data). However, the question as to whether this protein participates in thrombin-induced endothelial barrier dysfunction remains open. The actin-binding regulatory protein caldesmon undergoes PKC-mediated phosphorylation in thrombin-stimulated EC [80]. Caldesmon, which inhibits actomyosin interaction in the absence of calmodulin, can be directly phosphorylated by PKC *in vitro* [84]. It was shown that phosphorylation attenuates the inhibitory properties of caldesmon [84, 85]. Thus, in addition to Ca^{2+}/calmodulin, thrombin-induced activation of PKC may affect the properties of caldesmon and actomyosin contraction overall.

The intermediate filament protein vimentin is also phosphorylated by PKC in thrombin-stimulated EC [80]. It has been shown in *in vitro* experiments that vimentin filaments are disassembled during phosphorylation [86]. Further experiments are required to determine whether vimentin is rearranged in thrombin-stimulated EC and to what extent the stability of the intermediate filament network is crucial for endothelial barrier function.

It was also shown that a 15- to 30-min thrombin stimulation causes dissociation of cell–cell adhesion proteins p120, α- and β-catenin, and plakoglobin from VE-cadherin [9]. VE-cadherin is a transmembrane protein that is expressed only in the endothelium. It ensures Ca^{2+}-dependent junctions between adjacent cells in the adhesion regions. Catenins regulate VE-cadherin linkage to the actin cytoskeleton and formation of cadherin multimers. As a result of PKC inhibition in thrombin-stimulated cells, the complexes between VE-cadherin and p120, α- and β-catenin, and plakoglobin remain stable [9]; this suggests that PKC participates in thrombin-induced attenuation of cell–cell junctions.

ROLE OF cAMP IN THE REGULATION OF ENDOTHELIUM PERMEABILITY

Elevation of the intracellular cAMP concentration prevents the increase in permeability, formation of gaps between the cells, and formation of stress fibers in thrombin-stimulated EC [53, 87]. Overexpression of the endogenous PKA inhibitor PKI enhances thrombin-induced EC barrier dysfunction, whereas treatment of EC with pharmacological inhibitors of PKA stimulates stress fiber formation [87]. Similar to thrombin, PKA inhibitor induces translocation of MLCK and phosphatase 2B to F-actin [87]. The adenylate cyclase activator forskolin and cAMP analogs inhibit the thrombin-induced phosphorylation of MLC [53]. MLCK can be directly phosphorylated by PKA [78], which decreases MLCK affinity for calmodulin [88]. Actually, an increase in cAMP level in endothelium results in MLCK phosphorylation and decreases the activity of this enzyme [89]. In thrombin-stimulated EC, however, MLCK does not undergo dephosphorylation [58, 77] despite the decrease in cAMP concentration [32]. In addition, PKA inhibitor only slightly increases the level of basal and thrombin-stimulated MLC phosphorylation, which is suggestive of another mechanism of G_i-mediated increase in endothelial permeability in response to thrombin.

It has been shown that in nonmuscle cells PKA can phosphorylate the regulatory protein caldesmon [90]. However, the treatment of platelets with thrombin does not change the extent of caldesmon phosphorylation [90]. Another actin-binding G-protein, filamin, is also a target for PKA-mediated phosphorylation. Phosphofilamin is more resistant to proteolysis and more efficiently cross-links actin filaments [91]. However, cAMP-dependent phosphorylation of filamin and caldesmon remains to be confirmed or refuted.

It was shown that thrombin induces translocation of focal adhesion proteins paxillin and FAK to the sites of actin filament linkage. An increase in cAMP concentration in the cell blocks this translocation, whereas PKA inhibition in the absence of thrombin induces it [87]. In view of this, it is worth mentioning that VASP (vasodilator-stimulated phosphoprotein) not only actively participates in the actin cytoskeleton rearrangement, but also is a target for PKA [92]. In EC, VASP is located in the peripheral regions of the cells at the sites of cell–cell and cell–matrix junctions [93]. VASP can independently stabilize newly formed actin filaments or mediate this process via G-actin-binding G-protein profilin. In addition, matrix junction proteins zyxin and vinculin are also VASP ligands [92]. PKA-catalyzed phosphorylation inhibits VASP-induced nucleation of actin filaments. Interaction of VASP with profilin, vinculin, and zyxin is independent of PKA [92]. Such mode of VASP action suggests the hypothesis of cAMP-mediated regulation of cell–matrix junction formation. Both phosphorylated and dephosphorylated VASP forms complexes with zyxin and vinculin, which are linked to the transmembrane proteins integrins via talin. VASP-dependent actin polymerization, however, is triggered only by VASP dephosphorylation. Thus, decrease in cAMP con-

centration in the cell and PKA inhibition should induce the formation of cell adhesion sites and stress fibers linked to them [92].

As mentioned above, thrombin induces the dissociation of catenins from the cell–cell junction transmembrane protein cadherin [9]. As a result of thrombin stimulation, the membrane band of β-catenin going continuously along the EC peripheral region becomes thin and discontinuous [87]. An increase in cAMP concentration in EC markedly attenuates the thrombin-induced changes in β-catenin location. Conversely, PKA inhibition entails almost complete disappearance of β-catenin from EC peripheral regions in the absence of thrombin [87]. It is well known that β-catenin undergoes ubiquitin-dependent proteolysis and that its phosphorylation of glycogen synthase kinase GSK3β triggers its ubiquitination with subsequent degradation [94]. In addition, it was shown that the formation of the complex with cadherin stabilizes β-catenin [95]. It is unclear whether cAMP prevents β-catenin dissociation from cadherin and its possible degradation. However, the phenomenon of cAMP-dependent catenin stabilization in the membrane area is suggestive of the key role of PKA in this process.

TYROSINE KINASES AND PHOSPHATASES AND THEIR ROLE IN BARRIER DYSFUNCTION

More and more data indicate that tyrosine phosphorylation causes an increase in cell–cell junction permeability in the endothelium [96-98]. It has been shown that inhibitors of tyrosine protein kinases can attenuate the thrombin-induced increase in endothelial permeability [74, 97]. The role of tyrosine protein kinases Src and focal adhesion kinase (FAK) in the physiological response was studied in greatest detail. It was found that one of the endothelial MLCK isoforms can undergo phosphorylation by Src, which increases its activity [62]. Interestingly, the phosphatase 1 catalytic subunit can be phosphorylated and inhibited by Src [99]. Thus, tyrosine phosphorylation of MLCK and phosphatase 1 may be an additional mechanism that regulates MLC phosphorylation in EC. In the thrombin-stimulated EC, however, no increase in the extent of tyrosine phosphorylation of MLCK was observed [97]. As far as phosphatase 1 phosphorylation is concerned, it was shown that the inhibition of tyrosine protein kinases in EC results in the inhibition rather than activation of phosphatase activity [100].

Another potential Src substrate, cortactin, undergoes translocation in the thrombin-treated platelets and EC [101, 102]. Src-mediated phosphorylation attenuates the ability of cortactin to link the actin fibers *in vitro* [103]. Overexpression of cortactin unable to undergo phosphorylation by Src reduces the thrombin-induced stress fiber formation [102] and disrupts the ability of EC to migrate [104]. Whether cortactin and its phosphorylation contribute to the thrombin-induced endothelial dysfunction remains to be elucidated.

In umbilical vein EC, thrombin induces tyrosine phosphorylation of junctional proteins p120 and β- and γ-catenins [105]. Tyrosine phosphatase SHP2, which is associated with β-catenin in intact cells, is phosphorylated by Src 5 min after thrombin stimulation and dissociates from VE-cadherin complex, thus ensuring an increase in the extent of phosphorylation of the proteins contained in this complex [105]. With regard for all listed mechanisms of Src involvement in cytoskeletal rearrangement, it is interesting that in EC line EA.hy926 the inhibitors of Src were unable to suppress the thrombin-induced cell retraction, whereas conventional inhibitors of nonreceptor tyrosine protein kinases were sufficiently effective [45]. Whether this phenomenon is characteristic of all EC or only of EA.hy926 cells is still obscure.

As an Src substrate [106], FAK participates in tyrosine phosphorylation of several proteins, including paxillin [107]. However, although FAK catalyzes the phosphorylation of focal adhesion proteins, this phosphorylation is not required for the focal adhesion complex assembling [106, 108]. Overexpression in EC of FAK lacking the kinase domain reduces the ability of the cells to migrate and proliferate [108]. It was shown that thrombin stimulates tyrosine phosphorylation of paxillin and FAK [109]. Pretreatment of EC with the Rho inhibitor C_3-transferase completely abolishes the thrombin-induced increase in phosphorylation of these proteins [109].

OTHER EFFECTS OF THROMBIN ON ENDOTHELIUM

It was shown that thrombin causes the exposure of P-selectin on the EC surface [110]. This adhesion protein ensures the linkage and motility of polymorphous cells over EC and their penetration into the underlying tissues. In addition, thrombin induces the synthesis of platelet activation factor [111]. This phospholipid activates leukocytes, ensuring their junction to the endothelium and release of enzymes and reactive oxygen species. In addition, thrombin affects EC proliferation. It enhances the responses of umbilical vein EC to growth factors and has an independent mitogenic effect on capillary EC [25]. Thrombin also stimulates local secretion of endothelial chemoattractants and growth factors [112]. These effects may be of physiological importance, because the demand for new vessels often increases after clot formation or narrowing of vessel lumens caused by fibrin. Interestingly, fibrin is an optimal substrate facilitating EC attachment and proliferation [25]. Thus, clot formation entailing an increase in the local concentration of thrombin and fibrin is a potent angiogenic factor.

While the crucial role of thrombin in homeostasis and clotting was well studied long ago, the effect of thrombin on endothelium and the importance of this effect for the organism on the whole remained obscure for a long time. However, intravenously injected thrombin increases permeability of blood vessels, especially that of microcirculation vessels, resulting in pulmonary edema and heavy breathing. In addition, thrombin injection increases leukocyte content in lung alveoli. All these phenomena are due to the fact that thrombin contributes to endothelial dysfunction, disrupting the barrier function of EC monolayer and inducing inflammatory response. This review emphasizes the complexity of signal systems that regulate the equilibrium between the vascular space and underlying tissues and analyzes the key role of Ca^{2+}, MLCK, myosin phosphatase, Rho-kinase, and PKC, as well as their cytoskeletal targets in the thrombin-induced increase in endothelial monolayer permeability.

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