P2Y receptors as regulators of lung endothelial barrier integrity

Evgeny Zemskov[1], Rudolf Lucas[1], Alexander D. Verin[1,2], and Nagavedi S. Umapathy[1,2]

[1]Vascular Biology Center, [2]Pulmonary and Critical Care Medicine, Medical College of Georgia, Augusta, GA 30912, USA

Address for correspondence: *Dr. Nagavedi S. Umapathy, 1459 Laney Walker Blvd, Medical College of Georgia, CB 3701, Augusta, GA 30912, USA. E-mail: usiddaramappa@mcg.edu*

ABSTRACT

Endothelial cells (ECs), forming a semi-permeable barrier between the interior space of blood vessels and underlying tissues, control such diverse processes as vascular tone, homeostasis, adhesion of platelets, and leukocytes to the vascular wall and permeability of vascular wall for cells and fluids. Mechanisms which govern the highly clinically relevant process of increased EC permeability are under intense investigation. It is well known that loss of this barrier (permeability increase) results in tissue inflammation, the hall mark of inflammatory diseases such as acute lung injury and its severe form, acute respiratory distress syndrome. Little is known about processes which determine the endothelial barrier enhancement or protection against permeability increase. It is now well accepted that extracellular purines and pyrimidines are promising and physiologically relevant barrier-protective agents and their effects are mediated by interaction with cell surface P2Y receptors which belong to the superfamily of G-protein-coupled receptors. The therapeutic potential of P2Y receptors is rapidly expanding field in pharmacology and some selective agonists became recently available. Here, we present an overview of recently identified P2Y receptor agonists that enhance the pulmonary endothelial barrier and inhibit and/or reverse endothelial barrier disruption.

Key words: EPAC, lipopolysaccharide, microvascular endothelium, MLC-phosphatase, permeability, VE-cadherin

INTRODUCTION

The vascular endothelium is a semi-selective diffusion barrier between the plasma and interstitial fluid and is critical for normal vessel wall homeostasis. The endothelial permeability is regulated by the balance between centripetal and centrifugal intracellular forces, provided by the contractile machinery and the elements opposing contraction, respectively. The latter include tethering complexes, responsible for cell–cell and cell–matrix contacts, and systems granting cell rigidity and preventing cell collapse, such as actin filaments, microtubules, and intermediate filaments.[1] Some naturally occurring substances such as sphingosine-1-phosphate[2] and the second messenger cAMP[3] are known to enhance the endothelial barrier. Recently, much attention has been given to the therapeutic potential of purinergic agonists and antagonists for the treatment of cardiovascular and pulmonary diseases.[4,5] Purines and pyrimidines function as signaling molecules (receptor legends), which are released extracellularly from different sources in the body and subsequently reach the target organs.[6] Numerous published data obtained in *in vitro* and *in vivo* models suggest that they could be physiologically relevant factors protecting the endothelial barrier.[7,8] ATP, for example, can be released into the bloodstream from platelets[9] and red blood cells,[10,11] and its concentrations may temporarily exceed 100 μM.[12] Furthermore, the endothelium is a source of ATP locally within the vascular bed and ATP is released constitutively across the apical membrane of

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the endothelial cells (EC).[13] Enhanced release of ATP is observed from the EC in response to various stimuli, including hypotonic challenge,[13] calcium agonists,[13] shear stress,[14] thrombin,[14] ATP itself[15] and bacterial endotoxin, lipopolysaccharide (LPS).[16] Extracellular ATP may signal directly[17,18] and this signaling is mediated, in part, by P2Y purinergic receptors.[19,20]

Purine and pyrimidine receptors (simply called purinoceptors) are divided into two classes: P1 or adenosine receptors and P2, which recognize primarily extracellular ATP, ADP, UTP, and UDP.[6,21] The P2 receptors are further subdivided into two subclasses. P2X receptors are extracellular ATP-gated calcium-permeable nonselective cation channels that are modulated by extracellular Ca^{2+}, Na^{+}, Mg^{2+}, Zn^{2+}, and Cu^{2+}. Subtypes are defined according to the molecular structure of cloned mammalian P2 receptors, discriminated by different numerical subscripts (P2Xn or P2Yn). This forms the basis of a system of nomenclature that will replace the earlier subtype nomenclature (including P2X, P2Y, P2U, P2T, and P2Z receptors) as correlations between cloned and endogenous receptors are established.[22] Several studies demonstrated that P2X receptors are abundant in EC.[23–25] However, the P2X specific agonist, AMP-CCP, was completely inactive in human pulmonary artery endothelial cell (HPAEC)[26] and human lung microvascular endothelial cell (HLMVEC) monolayers (our unpublished data) suggesting that P2X receptors are unlikely to be involved in ATP-mediated pulmonary EC barrier enhancement/protection.

P2Y receptors are members of the G-protein-coupled receptors (GPCRs) superfamily, which consists of seven transmembrane domains, three extracellular and three intracellular loops, extracellular N- and intracellular C-termini. The receptors are coupled to their immediate effectors, heterotrimeric G-proteins, and function as guanidine exchange factors (GEFs). In the inactive state, heterotrimeric G-proteins are presented in the cell as αβγ trimers. Gα-subunit is dissociated from Gβγ dimer upon GTP binding and, as a result, two functionally-active effectors (Gα and Gβγ) emerge. Signaling cascades dependent upon Gα and Gβγ activation by P2Y receptors will be discussed further in the text. In mammalian cells of different origin, the expression of 39 distinct G-protein subunits has been documented: 21 α-subunits, 6 β-subunits, and 12 γ-subunits. These numbers suggest a remarkable variety of possible heterotrimer combinations. However, not all of them can be realized because of, for example, a tissue-specificity of some subunits.[27]

P2Y purinoceptors are activated by extracellular ATP/ADP/UTP/UDP-glucose/β-NAD. To date, eight P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) were identified in mammalian cells.[28,29] Expressions of P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, and P2Y14 purinoceptors had been shown in the endothelia or cultured EC.[23,29-32] Heterotrimeric G-proteins activated by P2Y receptors in the EC belong to four functionally distinct subfamilies: Gs, Gq/11, Gi, and G12/13. Activations of these particular G-proteins determine a cell response upon agonist stimulations. Here, we have focused on the effects of purine/pyrimidine-induced P2Y-mediated signaling on the endothelial integrity and respective cascades resulting an enhancement/loss of barrier function will be discussed.

SIGNALING PATHWAYS ACTIVATED UPON P2Y RECEPTOR STIMULATION

Endothelial integrity as well as endothelial barrier function is determined by cell–cell and cell–matrix contacts physically and functionally linking to the EC cytoskeleton. Purinoceptor-mediated signaling pathways affecting endothelial barrier function initiate dynamic changes in cytoskeleton organization, regulation of proteins linking cytoskeletal structures to adherens junctions (AJ), tight junctions (TJ), and focal adhesion (FA) contacts, protein components of AJ, TJ, and FA. AJ and TJ play an essential role in the endothelial cell–cell contacts. Vascular endothelial cadherin (VE-cadherin) is a major component of AJ, transmembrane protein involved in homotypic contacts with adjacent cells. Binding between extracellular domains of VE-cadherin molecules is Ca^{2+}-dependent, and a removal of Ca^{2+}-ions from cell culture medium lead to a quick disassembly of AJ and a loss of the EC monolayer integrity.[33,34] The cytoplasmic domain of VE-cadherin is linked to the cortical actin *via* β/α-catenins stabilizing AJ as such providing a basis for dynamic reorganization of cell–cell contacts. The EC TJ consists of transmembrane proteins claudins, occludins, and junctional adhesion molecules (JAM) linked to cytoplasmic proteins such as zonula occludens. Actin-mediated disassembly/stabilization of the cell–cell contacts can be determined by phosphorylation levels of actin-associated 20 kDa regulatory myosin light chain (MLC_{20}). The phosphorylation/dephosphorylation status of MLC_{20} plays an important role in actin cytoskeleton organization in the EC and therefore critical for endothelial barrier function.[35,36] Phosphorylation of MLC_{20} at its Thr-18/Ser-19 residues by Ca^{2+}/calmodulin-dependent MLC kinase (MLCK) or Rho kinase (ROCK) leads to actomyosin contraction, centripetal force-driven AJ and results in a loss of the EC monolayer integrity, intercellular

gap formation, and hyperpermeability.[37-39] By contrast, the pathways leading to dephosphorylation of MLC_{20} by MLC phosphatase (MLCP) or Ser/Thr protein phosphatase 1 (PP1), result in the formation of a thick cortical actin ring, cell relaxation and spreading. Highly-specific interaction between MLCP with its protein substrate, MLC_{20}, is determined by myosin phosphatase targeting regulatory subunit of PP1 (MYPT1), the regulatory subunit of PP1. Moreover, an interaction of MLCP and MLC_{20} can be abolished, if MYPT1 is phosphorylated by ROCK at Thr-696/Thr-850.[40-42] This inhibitory modification of MYPT1 prevents MLCP-dependent dephosphorylation of MLC_{20} and therefore has a negative effect on the barrier function amplifying F-actin stress fiber formation. As generally considered, an activation of small GTPase RhoA is crucial for the endothelial hyperpermeability. Expression of constitutively active RhoA in the EC is sufficient to induce the monolayer integrity loss.[43] Furthermore, various edemagenic factors (such as thrombin, vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), lysophosphatidic acid (LPA), microtubule destabilizers (nocodazole, 2-methoxyestradiol), etc.) were shown to compromise the endothelial barrier by a RhoA-dependent mechanism[43-46] and an inhibition of either RhoA or its effector, ROCK, could significantly protect the barrier function of the challenged EC.[44,46]

In the EC, agonist-mediated activation of P2Y receptors may enhance or decrease a barrier function of the endothelium [Figure 1]. Stimulated P2Y11 receptor promotes Gs protein activation,[47-50] direct interaction of free Gαs-subunit with plasma membrane adenylate cyclase (AC), and elevation of cAMP levels in targeted cells.[51] Numerous publications indicate that the second messenger cAMP has a critical role in a positive modulation of the barrier function.[52-57] The cAMP-dependent activation of protein kinase A (PKA) has indispensible consequences as a potent positive regulator of endothelial integrity. Recently published data suggest that PKA may prevent RhoA activation by phosphorylation of RhoGDI at Ser-174[58] and stimulate MLCP via phosphorylation of MYPT1 at Ser-695,[39] shifting the EC to the relaxed shape by prevention of MLC_{20} phosphorylation and stress fibers formation. Besides, a generation of cAMP may lead to alternative, PKA-independent activation of Exchange Protein directly Activated by cAMP (Epac1) and its down-stream effectors, Rap1 and Rac1.[52,53,55-57,59,60]

Purinoceptor P2Y14 is involved in heterotrimeric Gi-protein-mediated signaling, which results in an interaction of free Gαi-subunit with AC and inhibition of cAMP synthesis.[38,61] Besides, Gi-protein-derived Gβγ-dimers

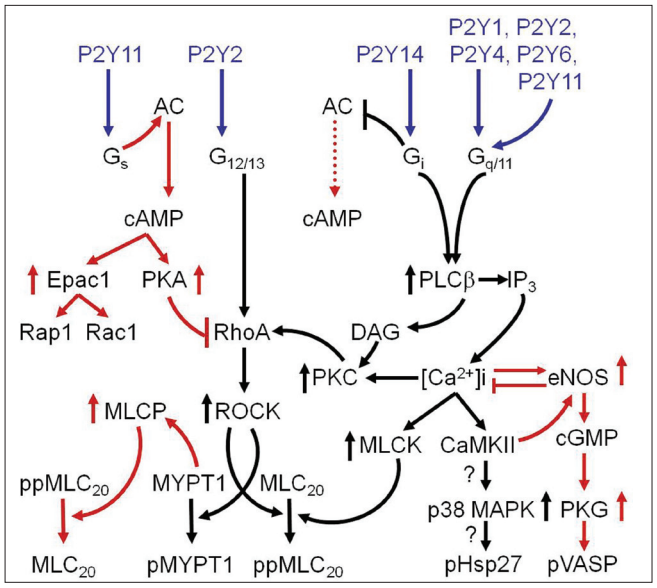

Figure 1: Schematic representation of the P2Y-receptor-activated signaling network in ECs. Potential endothelial barrier-protective and barrier-disruptive pathways are shown by red and black arrows, respectively (see detailed explanation in the text)

initiate PI3-kinase (PI3-K) or phospholipase Cβ (PLCβ) signaling pathways.[28,62,63] PI3-K activates PKB/Akt[64] and ERK1/2.[65] PLC activation results in elevation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) levels and may follow by $[Ca^{2+}]_i$ influx due to stimulation of plasma membrane and endoplasmic reticulum Ca^{2+}-channels.[63] Elevation of $[Ca^{2+}]_i$ and DAG levels can induce activation of several PKC isoforms.[66] In case of the regulation of RhoA/ROCK signaling, the PKCα isoform functions as a PKA antagonist, since it may activate this pathway by direct phosphorylation of the upstream effectors, RhoGDI and RhoGEF,[67] increasing, therefore, MLC_{20} phosphorylation. PKCα may also regulate AJ disassembly via phosphorylation of p120 and β-catenin.[68] P1-purinoceptor-mediated activation of Gαi subunits has also been shown to promote an upregulation of p38 MAPK[65] and may possibly activate JNK by ROCK-dependent phosphorylation[69]; however, these pathways were not described for P2Y-mediated Gi-signaling. The p38 MAPK can initiate stress fiber formation via phosphorylation of actin-capping protein hsp27 and its further dissociation from actin filaments.[8,70] Another important event related to Gi protein-mediated signaling is an activation of Src protein tyrosine kinase (PTK).[71,72] In the EC, Src family PTK may modulate the barrier function by tyrosine phosphorylation of major protein components of AJ and TJ, although the effect of such phosphorylation on endothelial permeability still needs to be clarified.[73-75]

Gq/11-protein-mediated signaling is activated by agonist stimulation of P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11

receptors.[47,48,76-81] Free Gαq or Gα11 interact with PLCβ and enhance synthesis of IP_3 and DAG.[63] This essentially results in $[Ca^{2+}]_i$ influx and activation of PKC isoforms.[50] Extensive studies performed in the EC have demonstrated Ca^{2+}-dependent activation of endothelial nitric oxide (NO) synthase (eNOS) (via direct interaction with Ca^{2+}/calmodulin and/or via phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)).[82,83] NO stimulates guanylate cyclase (GC), resulting in an elevation of second messenger cGMP levels and cGMP-dependent protein kinase G (PKG) activation.[84] This pathway serves as a negative feedback control of Ca^{2+} influx through down-regulation of endoplasmic reticulum (ER) IP_3-sensitive channels and plasma membrane Ca^{2+}-influx channels[85,86] and increases Ca^{2+} uptake by ER via activation of ER Ca^{2+} ATPases.[85] Thus, eNOS/GC/PKG pathway can down-regulate the barrier-compromising Ca^{2+}-mediated cell signaling. In human umbilical vein EC (HUVEC), stimulatory phosphorylation of eNOS at Ser-1177 can be activated by extracellular ATP, UTP, or ADP. Inhibitory analysis suggested an involvement of P2Y1, P2Y2, and, possibly, P2Y4 receptors in the activation of eNOS via $[Ca^{2+}]_i$ increase and DAG-dependent PKCδ.[87] Another protein target of activated PKG is vasodilator-stimulated phosphoprotein (VASP), a protein regulating actin polymerization.[88] PKG/PKA-phosphorylated VASP has been detected in endothelial cell–cell junctions (TJ and AJ).[89,90] Although an entire role of VASP phosphorylation in endothelial contraction/relaxation remains unclear, this modification correlates with an enhancement of the barrier function in P2Y agonist-stimulated EC monolayers.[26,89]

P2Y2 receptor can also activate Gα12-dependent pathways. This signaling requires an interaction of the purinoceptor with αvβ3-integrin, since it can be inhibited either by αv-integrin antisense oligonucleotides or by point mutation in an integrin-binding sequence of the P2Y2 receptor.[91] Activation of G12 protein positively modulate Rho-guanine nucleotide exchange factor (p115Rho-GEF) via its interaction with Gα12 subunit[92] or by activated PKCα phosphorylation.[93] This, in turn, can promote RhoA-dependent ROCK activation and phosphorylation of MLC_{20} and MYPT1.

Elevation of cytosolic Ca^{2+} in the EC is a common consequence of activation of most P2Y receptors coupled to Gs (via cAMP-activated Ca^{2+}-channels), Gq/11 and Gi (via IP_3-mediated Ca^{2+} release). $[Ca^{2+}]_i$ is essential for activation of eNOS and endothelium-derived release of vasorelaxant, NO,[94,95] however, an elevation of cytosolic Ca^{2+} is certainly a negative factor for endothelial integrity. Nevertheless, in extracellular purine-activated EC, Ca^{2+} influx is a transient and its effect does not overcome the barrier enhancement.[26,96]

P2Y RECEPTORS EXPRESSION ANALYSIS IN PULMONARY ENDOTHELIUM

Earlier studies indicated that the most abundant P2 receptor in EC is P2X4[25] and the other study indicates that P2X4, P2Y11, P2Y1, and P2Y2 are the most expressed P2 receptors in HUVEC.[23] However, in rabbit pulmonary artery EC, the mRNA expression analysis indicates that P2Y1, P2Y2, and P2Y4 receptors are abundantly expressed, but not P2Y6 receptors.[97] Since the expression pattern of P2Y receptors in pulmonary ECs has not been reported earlier, we have used highly clinically relevant human EC to study the mRNA expression. Our quantitative Real-Time RT-PCR (qPCR) analysis of P2Y mRNA expression identifies mRNA for P2Y1, P2Y2, P2Y11, P2Y12, and P2Y14 receptors in both macro (HPAEC) and micro (HLMVEC) vascular pulmonary EC [Figure 2]. Interestingly, P2Y receptors expression levels is quite different in these two closely related cell types of pulmonary vasculature. The P2Y11 receptor (coupled to both Gq and Gs) was highly expressed, P2Y14 receptor expression was moderate and the other P2Y receptors (P2Y1, Y12, and Y2) expression was low to very low levels in HPAEC. However, the mRNA expression levels P2Y receptors were quite different in HLMVEC compared to HPAEC and they all distributed quite significantly [Figure 2]. Our results suggest that P2Y receptors signaling are different in these closely related cell types and detailed studies with receptor-specific agonists and antagonists are needed in order to develop P2Y receptor-based therapeutics.

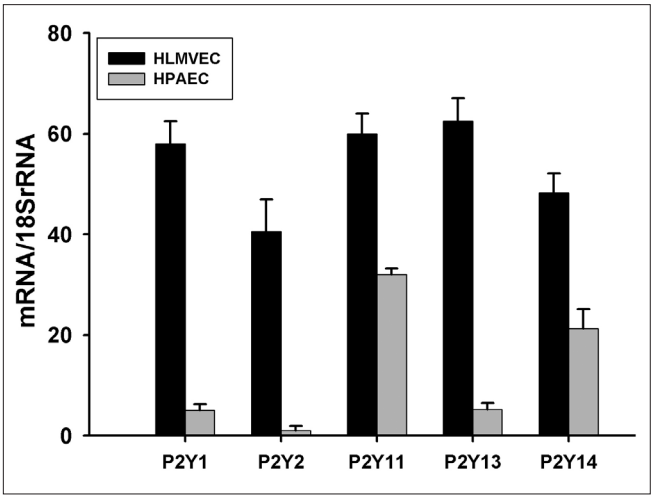

Figure 2: Quantitative Real-Time PCR analysis of the P2Y receptors mRNA expression in both HPAEC and HLMVEC. The P2Y receptor expression was normalized with 18S ribosomal RNA

ROLE OF P2Y RECEPTORS IN PULMONARY EC BARRIER ENHANCEMENT

Since multiple P2Y receptors are expressed at various levels on the pulmonary EC [Figure 2], it is essential to emphasize the P2Y receptor(s) responsible for the pulmonary endothelial barrier enhancement and protection against various insults. The possible interactions between naturally occurring receptor agonists and P2Y receptors expressed on pulmonary EC are very complex. The data regarding role of purines and pyrimidines in the maintenance and alteration of EC barrier are contradictory. Barrier-protective property of ATP has been reported.[96,98] On the other hand, P2Y1-receptor agonists, 2-methylthio ATP (2meS-ATP) and ADP decreased cell size and enhanced permeation of FITC-labeled dextran through HUVEC monolayers.[99] ATP was found to increase paracellular permeability of microvascular endothelium in frog microvessels.[99,100] Our studies demonstrate that ATP and its stable analogs significantly increase the transendothelial resistance (TER) in highly clinically relevant human pulmonary EC via P2Y receptors.[26] Recent studies showed that β-nicotinamide adenine dinucleotide (β-NAD), an important co-enzyme for cellular metabolism, is an important vascular mediator,[101,102] elicits cellular effects through activation of P2Y1/Y11 receptors.[103,104] In addition to ATP, the β-NAD secreted extracellularly from endothelium.[105] Our recent studies demonstrated that extracellular β-NAD significantly enhances the pulmonary endothelial barrier in a dose-dependent manner via P2Y receptors.[80]

SIGNIFICANCE OF SPATIAL DISTRIBUTION P2Y RECEPTORS IN ENDOTHELIUM AND ITS RELEVANCE TO THE BARRIER PROTECTION

We have shown that various P2Y receptors are expressed in pulmonary EC at various levels [Figure 2]. However, the expression levels of these receptors on apical and basal side of the pulmonary endothelium are not known. We speculate that extracellular purines and pyrimidines released from the blood cells (for example, platelets), apical side of EC or alveolar epithelial cells (basal side of EC) stimulate P2Y receptors based on their expression pattern (apical or basal). In addition, a recent study indicated the hetero-oligomerization between two metabotrofic purinoceptors, P2Y1 and P2Y11, co-expressed in HEK293 cells, promotes agonist-induced internalization of the P2Y11 receptor, which itself is unable to undergo endocytosis.[106] Moreover, the agonist profile for the co-expressed P2Y1 and P2Y11 was different from the agonist profile established for cells expressing the P2Y11 receptor only. The hetero-oligomerization of the P2Y1 and P2Y11 receptors modifies the functions of the P2Y11 receptor in response to extracellular nucleotides. Further, a recent study indicate that the human bronchial epithelia express P2Y6 receptors on both apical and basolateral membranes and that the cAMP/PKA pathway regulates apical but not basolateral P2Y6 receptor-coupled ion transport.[107] Therefore, selective activation of specific P2Y receptors responsible for barrier protection might form a basis for the treatment of various lung disorders. The therapeutic potential of P2Y receptors is rapidly expanding field in pharmacology and some selective agonists became recently available.

Table 1 represents both native and synthetic P2Y agonists and antagonists that were used to study P2Y receptors. The P2Y agonist or antagonist can be purchased from Sigma-Aldrich (St. Louis, MO) or Tocris Biosciences (Ellisville, MO). The pharmacological armamentarium for P2Y receptors is limited and agonists that exhibit high-affinity selectivity among P2Y receptors as well as resistance to ectoenzyme-catalyzed metabolism are few. More studies

Table 1: List of P2Y receptors agonist/antagonist (native and synthetic)

P2Y receptors	Native/Synthetic	
	Agonist	Antagonist
Y1	MRS 2179, MRS 2365, 2-MeSADP, ADP, (3-NAD	MRS 2500, MRS 22279, MRS 2179
Y2	UTP, ATP, UTPyS, MRS 2768	AR-C 126313, Suramin
Y4	UTP, UTPyS	ATP, Suramin
Y6	UDP, UTP, UDP(3S, PSB 0474	MRS 2578
Y11	BzATP, ATPyS, ATP, (3-NAD	NF 157, NF 340
Y12	2-MeSADP (b), ADP (a), ATP (a)	ARC 66096, 2-MeSAMP
Y13	2-MeSADP, ATP, 2-MeSATP	MRS 2211
Y14	UDP-glucose, UDP-galactose, UDP-N-acetyl-glucosamine, MRS 2690	

are needed in order to characterize the agonist profile of expressed multiple P2Y receptors on the apical or basal side of EC and the pathophysiological agonist concentrations that selectively activate P2Y receptors. Future detailed studies including expression analysis on both apical and basal EC membrane will help to establish conditions for possible P2Y receptor based therapies.

PROTECTIVE EFFECTS OF P2Y RECEPTORS MEDIATED SIGNALING AGAINST BACTERIAL TOXINS-INDUCED PULMONARY EC HYPERPERMEABILITY

The pulmonary EC lining the vessels are in contact with each other and render the vascular wall into a tight barrier. Any breach in the EC barrier results in leakage of fluid from the lumen of the vessels into the interstitial tissue and/or alveolar lumen, severely impairing gas exchange. Disruption of the vascular barrier is a prominent feature of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) syndrome and results in pulmonary edema formation and subsequent respiratory dysfunction or failure.[108-110] The barrier-compromising mechanisms of bacterial toxins (for example, Gram-negative endotoxin, LPS) revealed in *in vitro* models of pulmonary endothelial dysfunction have been demonstrated to target the actin cytoskeleton inducing actin stress fiber formation and intercellular gaps.[54,111] An essential step of such remodeling was an activation of MLC_{20}-specific protein kinases, namely, ROCK and nonmuscle MLCK.[112,113] As the result, mono- and di-phospho-MLC_{20} could be detected in LPS-treated cultured EC and in lung tissue of LPS-challenged mice. Significance of activation of the MLC_{20}-kinases for the endothelial barrier integrity *in vivo* was confirmed in murine model of ALI (intratracheal instillation of LPS). Inhibitors of ROCK[113] and MLCK,[112] as well as depletion of MLCK expression *in vivo*,[112] significantly attenuated the barrier dysfunction. These findings formed a basis for an application of the endothelium-protective agents such as agonists of P2Y purinoceptors. Indeed, the agonists stimulating heterotrimeric Gs-protein-coupled purinoceptors activate AC and elevate cAMP levels in the EC have been considered as the barrier protectors, since cAMP could reverse the barrier-compromising effects.[54,80,114] However, activation of heteromeric G-proteins Gq and Gi2 may also to be barrier-protective upon extracellular ATP stimulation.[26] Our recent studies with agonists of P2Y receptors (ATP, ATPγS, and β-NAD) in a clinically relevant HPAEC[26,80,115] and a murine model of LPS-induced ALI[115] demonstrate the barrier protection. Despite the obvious importance of the EC barrier, significant information concerning its regulation is still lacking. Furthermore, a paucity of information exists concerning the mechanisms involved in preservation of barrier integrity. Therefore, novel strategies to protect the EC barrier could have a profound clinical impact.

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