Purinergic Regulation of Endothelial Barrier Function

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Abstract: Increased vascular permeability is a hallmark of several cardiovascular anomalies, including ischaemia/reperfusion injury and inflammation. During both ischaemia/reperfusion and inflammation, massive amounts of various nucleotides, particularly adenosine 5′-triphosphate (ATP) and adenosine, are released that can induce a plethora of signalling pathways via activation of several purinergic receptors and may affect endothelial barrier properties. The nature of the effects on endothelial barrier function may depend on the prevalence and type of purinergic receptors activated in a particular tissue. In this review, we discuss the influence of the activation of various purinergic receptors and downstream signalling pathways on vascular permeability during pathological conditions.

Keywords: Rac1; RhoA; peripheral actin; adenosine; ATP; ADP; UTP; endothelial permeability; oedema; P2X receptors; P2Y receptors

1. Introduction

The vascular endothelium (VE), consisting of monolayers of endothelial cells (ECs), is located at the interface between the vascular and perivascular compartments and extends over a wide surface area. The VE separates strictly two compartments and regulates the trafficking of ions, solutes, macromolecules and leukocytes across the vessel wall, thus maintaining tissue homeostasis [1,2]. Additionally, it secretes several vasoactive agents that not only maintain its integrity but also regulate platelet function and vascular smooth muscle tone, and thus actively participate in the regulation of blood pressure. The semipermeable barrier function of VE is dependent on the size of the molecules, and this size-selective nature of the barrier to plasma proteins is a key factor in establishing protein gradients, which is required for fluid balance of tissues [1,3]. The loss of this barrier function of VE results in increased vascular permeability and leakage of blood components, which may finally result in organ dysfunction and life-threatening oedema formation [2,4].

Endothelial barrier integrity is maintained by the equilibrium of competing adhesive and contractile forces generated by adhesive molecules located at cell–cell and cell–matrix contacts and the acto-myosin-based contractile machinery, respectively [5]. ECs are tightly interconnected by the interaction of junctional proteins such as VE-cadherin, zona occludin 1 (ZO-1), occludins, and catenins that are linked to the actin cytoskeleton of adjacent cells [6,7]. Therefore, changes in the actin cytoskeleton dynamics and/or activation state of the EC contractile machinery may affect the stability of cell–cell junctions and barrier function.
Two members of the Rho family of GTPases, RhoA and Rac1, are the major regulators of endothelial actin cytoskeleton dynamics and contraction and thereby play a key role in the maintenance of endothelial barrier integrity. Constitutive activation of RhoA results in the loss of basal VE-cadherin and potentiates hypoxia-reoxygenation (H/R)-induced loss of endothelial barrier function, whereas suppression of RhoA activity attenuates the agonist-induced increase in endothelial permeability [8,9]. On the other hand, suppression of Rac1 activity in cultured ECs results in loss of the endothelial barrier and abolishes the recovery of EC barrier integrity following H/R-induced barrier failure. Accordingly, constitutive activation of Rac1 results in strong junctional staining of VE-cadherin and abrogates H/R-induced loss of cell–cell junctions [8].

The activation state of the endothelial contractile machinery is regulated by the phosphorylation state of regulatory myosin light chains (MLC), which are phosphorylated by MLC kinase (MLCK) [10] and dephosphorylated by MLC phosphatase (MLCP) [11]. Activation of Rho/Rho kinase (Rock) and MEK/ERK pathways induces MLC phosphorylation via inhibition of MLCP or activation of MLCK, respectively [12–14]. Thrombin inhibits MLCP by inducing the phosphorylation of its regulatory subunit MYPT1 at T850 and activates MLCK via phospholipase C/inositol tris-phosphate (PLC/IP3)-dependent release of Ca$^{2+}$ from intracellular stores [12,14,15]. Both of these actions contribute to its endothelial barrier destabilisation properties. A schematic presentation of mechanisms regulating endothelial barrier properties is shown below (Figure 1).

![Figure 1. Schematic presentation of regulators of endothelial barrier properties. Rock: Rho associated kinase](image)

Endothelial barrier integrity is influenced by several circulating, blood-borne hormones and agents/factors such as adenosine triphosphate (ATP) and its metabolites adenosine diphosphate (ADP) and adenosine. The major sources of vascular nucleotides are erythrocytes, platelets, and the endothelium [16]. Platelets contain nucleotides in their granules, and upon degranulation, bulk plasma levels of ATP can reach 50 µM [17], with even higher local concentrations predicted at the endothelial surface [18]. The endothelium releases nucleotides in response to shear stress [19], inflammatory mediators like thrombin [20], and hypoxia [21,22]. ATP and other nucleotides either released from vascular cells or applied exogenously can act at endothelial purinoceptors and modulate the barrier function of the endothelium [21,23,24]. Activation of purinergic receptors also induces the release of von Willebrand Factor (vWF) from ECs [25], which via reactive oxygen species (ROS)-dependent upregulation of endothelin-1 [26] may modulate endothelial barrier function.
2. Purine Receptors

There are two main classes of purine receptors: P1 receptors activated by adenosine and analogues, and P2 receptors recognised by purine and pyrimidine nucleotides (ATP, ADP, uridine triphosphate (UTP), uridine diphosphate (UDP)). P1 receptors are further divided into A1, A2, and A3 subtypes, depending on their affinity for adenosine. P2 receptors are further classified into ionotropic P2X and metabotropic P2Y receptors [27,28]. Nineteen different human purine receptors have been identified, cloned, and characterised [29]. Nearly all of these receptors are expressed on various cells of the cardiovascular system [27,29]. Several types of cells, particularly ECs and platelets, actively release nucleotides such as ATP that can activate a variety of the purine receptors in the vicinity [30,31]. This receptor activation scheme may be further complicated by the activity of ectonucleotidases that hydrolyse ATP to adenosine, which can activate P1 receptors [32].

3. Adenosine and Adenosine (P1) Receptors

Physiological extracellular adenosine levels range from 20 to 300 nM, which rise to a low micromolar range during exercise and to a high micromolar level under pathological conditions like ischaemia [33,34]. Under physiological conditions, the major source of extracellular adenosine is intracellular adenosine released by nucleotide transporters; however, under stress conditions, it is generated from its precursors ATP, ADP, and adenosine monophosphate (AMP) by the combined activities of extracellular ectonucleotidases, CD73 and CD39 [35]. Extracellular adenosine mediates its effects via adenosine receptors. There are four well-characterised adenosine receptors, namely adenosine A1, A2A, A2B, and A3, which are classified as high (A1, A2A, A3) or low (A2B) affinity for binding their parent physiological agonist, adenosine [36]. All four adenosine receptors possess seven transmembrane domains and belong to the family of G-protein-coupled receptors (GPCR) [37]. The A1 and A3 receptors are coupled to Go and/or Gi/o, whereas A2A and A2B are coupled to Gs proteins. Activation of A1 and A3 receptors results in inhibition of adenylyl cyclase (AC) activity, leading to reduction in cyclic AMP (cAMP) production and suppression of downstream signalling [37,38]. Their activation also leads to PLC/IP3-dependent release of Ca2+ from the endoplasmic reticulum (ER), protein kinase C (PKC) activation, and nitric oxide (NO) production [39–42]. In cardiomyocytes and neurons, activation of A1 adenosine receptors stimulates the opening and blockade of K+ channels and P- and N-type Ca2+ channels, respectively [43,44]. Activation of both A1 and A3 receptors leads to PKC-dependent and independent mitogen-activated protein kinase (MAPK) activation [45]. Activation of both adenosine A2A and A2B receptors results in activation of AC, enhanced cAMP production, and activation of downstream signalling [36]. Adenosine receptors are widely distributed throughout the nervous, cardiovascular, respiratory, urogenital, gastrointestinal, and immune systems. All adenosine receptors are expressed on various cells of the cardiovascular system, including ECs [37,46].

3.1. Adenosine Receptors and the Endothelial Barrier

Adenosine is a non-selective agonist for all adenosine receptors and produces differential effects on endothelial permeability of various vascular beds depending on the type of receptors expressed.

3.1.1. Adenosine Receptors and Lung Microvascular Permeability

In the lung vasculature, adenosine signalling has largely been shown to enhance endothelial barrier properties and ameliorate agonist-induced hyperpermeability. In a mouse model of acute lung injury, knockdown of CD39 or inhibition of CD73, the two sequential enzymes responsible for adenosine production, resulted in development of severe lung oedema in response to ventilation compared with wild-type littermates. These animals were rescued by the addition of exogenous apyrase, suggesting a protective role played by adenosine [47]. Both adenosine A2A or A2B receptors seem to mediate the protective effects of adenosine in the lung [48,49]. In an isolated rat lung perfusion model...
of ischaemia/reperfusion (IR), a selective A$_2$-receptor agonist reduced the IR-induced increase in microvascular permeability [50]. Pharmacological activation of adenosine A$_{2A}$ and A$_{2B}$ receptors protected against hypoxia and lipopolysaccharide (LPS)-induced development of lung oedema [51,52], whereas deletion of adenosine A$_{2A}$ or A$_{2B}$ receptors in mice resulted in loss of adenosine-mediated preservation of the lung microvascular endothelial barrier [51,52]. These protective effects are mediated via augmented production of cAMP and downstream activation of Rac1 [53]. Likewise, we have previously shown that elevation of intracellular cAMP via adrenomedullin receptor activation protects against lung oedema [54]. On the other hand, too much adenosine also seems to be detrimental for the lung vasculature. Deletion of adenosine deaminase, an enzyme responsible for adenosine degradation, resulted in severe respiratory distress and lung inflammation in mice [55]. However, deletion of A$_{2B}$ receptors in these mice did not rescue but worsened the conditions, which were accompanied by enhanced loss of pulmonary barrier function [56], suggesting a protective role of A$_{2B}$ receptors. In contrast to murine lungs, in feline lungs, adenosine A$_1$ receptor activation mediates IR- and LPS-induced pulmonary microvascular barrier disruption [57,58]: perfusion with A$_1$ receptor antagonists xanthine amine congener (XAC)/8-cyclopentyl-1,3-dipropylxanthine (DPCPX) ameliorates IR-induced lung injury and oedema in these animals. These species differences are probably due to differential expression of adenosine receptors in murine and feline lungs. Like A$_2$ receptor activation, pharmacological activation of adenosine A$_3$ receptors with a selective agonist also protects against reperfusion-induced lung oedema. This protective effect is lost in A$_3$ knockout mice in vivo [59]. However, the mechanism of this protective effect is still elusive.

3.1.2. Adenosine Receptors and the Blood–Brain Barrier

The blood–brain barrier is a highly specialised structure formed by a very tight monolayer of microvascular ECs that are distinct from ECs of other vascular beds [60]. The brain ECs form tight junctions consisting of claudins, occludins, VE-cadherin, junctional adhesion molecules (JAMs), and zonula occludens (particularly ZO-1). Human and murine brain microvascular ECs express adenosine A$_1$ and A$_{2A}$ receptors [61–63]. Adenosine causes an elevation of central nervous system (CNS) barrier permeability. In an elegant study, Carman et al. demonstrated that a stable adenosine analogue 5’-N-ethylcarboxamidoadenosine (NECA) and selective A$_1$ and A$_{2A}$ receptor agonists increased blood–brain barrier permeability to low-molecular-weight dextran [63]. These adenosine effects were attenuated in mice lacking either A$_1$ or A$_{2A}$ receptors [63]. Similarly, mice lacking CD73 had low levels of extracellular adenosine and were protected against experimental autoimmune encephalomyelitis-induced development of brain oedema and leukocyte infiltration [64]. Accordingly, inhibition of endothelial A$_{2A}$ receptors protected mice against thromboembolic stroke-induced development of cerebral oedema and leukocyte infiltration [65]. Likewise, regadenoson, a selective A$_{2A}$ receptor agonist used clinically as a coronary vasodilator for myocardial perfusion imaging, increased permeability of the human blood–brain barrier in vitro [66] and in that of the mouse in vivo [63]. It has recently been shown that certain viruses and bacteria exploit this reaction of the blood–brain barrier to adenosine to open the barrier for their entry into the brain by increasing local production of adenosine, which causes transient opening of the blood–brain barrier and allows their entry to the central nervous system (CNS) [67,68]. Several groups have also recently tried to exploit this property of adenosine receptor activation to transiently open the blood–brain barrier for the local delivery of drugs to the CNS [69–74].

3.1.3. Adenosine Receptors and Coronary Microvascular Barrier

As in the blood–brain barrier, adenosine receptor activation in the coronary microvasculature results in loss of barrier integrity. A$_2$ receptor activation increased permeability of rat coronary microvascular ECs in vitro [75]. Infusion of adenosine in pigs on a high-fat diet resulted in increased cardiac microvascular permeability in vivo [76]. Similarly, Di Napoli et al. showed that DPCPX abrogates reperfusion-induced coronary hyperpermeability [77].
However, the authors used DPCPX at a concentration that blocks all adenosine receptors, suggesting A_2 receptors were also antagonised. In line with these reports, we have previously demonstrated that reperfusion caused the release of ATP from isolated rat coronary microvascular ECs that was degraded to adenosine. Inhibition of either ectonucleotidases or adenosine receptors abrogated endothelial barrier failure, whereas addition of apyrase and ectonucleotidases worsened reperfusion-induced endothelial barrier failure [21]. In a follow-up study, we demonstrated that adenosine induced an increase in rat mesentery microvascular permeability in situ and cardiac oedema in vivo. These adenosine effects were blocked by adenosine receptor antagonists. Furthermore, we showed that these effects were due to cAMP-mediated disruption of the microvascular endothelial cytoskeleton [78]. In a related study, we demonstrated that adenosine induced cAMP production (via adenosine A_2 receptors) in coronary microvascular ECs [79] that caused an inhibition of RhoA and Rac1 signalling [80]. This is in contrast to macrovascular ECs, where cAMP production inhibited RhoA/Rock signalling while activating Rac1 GTPase [81,82]. Inhibition of both RhoA and Rac1 results in complete breakdown of the EC cytoskeleton and disruption of cell–cell junctions [78,80]. Activation of Rac1 rescued these cells from the loss of endothelial barrier integrity [80].

3.1.4. Adenosine Receptors and the Macrovascular Endothelial Barrier

In general, adenosine receptor activation in macrovascular ECs enhances endothelial barrier properties and ameliorates the effect of barrier-disrupting agents [79,83–85]. The mechanism involves the production of cAMP via activation of A_2A and A_2B receptors by adenosine and its analogues. Enhanced cellular cAMP levels suppress the activity of the endothelial contractile machinery in a RhoA/Rock-dependent manner and activate Rac1 GTPase via protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) activation [82]. Table 1 summarises the major preclinical studies that investigated purinergic receptors in relation to endothelial barrier function, and Figure 2 summarises the key mechanisms involved in the adenosine receptors-mediated endothelial barrier regulation in various vascular beds.

Table 1. Effect of purinergic receptor activation/inhibition on endothelial barrier of various vascular beds.

| Receptor/Agonist | Model | Observation | Reference(s) |
|------------------|-------|-------------|--------------|
| Adenosine        | CD39 KO mice | Lung oedema | [47] |
| ATP + Apyrase    | Rat heart perfusion in vivo | Increased oedema | [78] |
| A_1 antagonist   | Feline lung (IR) in vivo | Reduced lung oedema | [57,58] |
| A_1 and A_2A KOsA_1 and A_2A agonists | Mouse BBB in vivo | A_1/A_2A agonists induced BBB permeability, effects lost in KOs | [63,66] |
| FDA approved A_2A agonist regadenoson | Rat model of brain drug deli-very | Increased BBB permeability of test drugs | [69,70,72,73] |
| A_2A agonist    | Isolated pig lungs (IR) | Reduced lung oedema | [48] |
| A_2A KO/A_2A agonist | Lung permeability in vivo | A_2A agonist reduced lung permeability/Effect lost in A_2A KO | [51] |
| A_2B KO         | Ventilator-induced lung injury | Increased lung oedema | [49] |
| A_3 KO/A_3 agonist perfusion | Lung IR (oedema) in vivo | A_3 agonist reduced lung oedema/Effect lost in A_3 KO | [86] |
| P2X4 antagonist | Brain middle artery occlusion (IPC-IR) mouse model | P2X4 antagonist abrogates protective effects of IPC | [87] |
| P2X7 antagonists | Rat intracranial haemorrhage/oedema | P2X7 antagonists alleviate oedema development | [88] |
| P2X7 KO         | Mouse traumatic brain injury | Reduced oedema development in KOs | [89] |
| P2X7 KO         | Mouse middle cerebral artery occlusion | Aggravated oedema development in KOs | [90] |
| P2Y_1/apoE double KO | Atherosclerosis | Reduced atherosclerotic plaques in double KOs | [91] |
| P2Y_1 agonist   | Mouse traumatic brain injury | P2Y_1 agonist ameliorates oedema development | [92] |
Table 1. Cont.

| Receptor/Agonist Model | Model | Observation | Reference(s) |
|------------------------|-------|-------------|--------------|
| EC-specific P2Y2/apoE double KO | Atherosclerosis | Development of stable plaques in double KOs | [93] |
| P2Y4 KO | Myocardial infarction | Protection against myocardial infarction injury | [94] |
| P2Y6/apoE double KO | Atherosclerosis | Double KOs develop smaller and less inflamed lesions | [95] |
| P2Y12 antagonist | In vitro endothelial barrier model | P2Y12 antagonist ameliorates thrombin-induced hyperpermeability | [84] |

ATP: adenosine 5'-triphosphate; BBB: Blood–brain barrier; EC: endothelial cell; FDA: United-States food and drug administration; IR: Ischaemia reperfusion; IPC: Ischaemic pre-conditioning; KO: Knockout.

Figure 2. Key mechanisms involved in adenosine receptors-mediated endothelial barrier regulation.

In lung microvasculature and macrovascular endothelium, A2 receptor activation causes an activation of Rac1 and an inhibition of RhoA, leading to stabilisation of the endothelial barrier. On the other hand, in coronary microvascular ECs, inhibition of both RhoA and Rac1 results in disruption of endothelial cytoskeleton and barrier failure. Black arrows indicate sequence of signal transduction, broken arrow indicates involvement of multiple steps in between, and green arrows indicate increase in cellular levels of indicated second messenger. Red bocks mean inhibition. AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; GEF: guanine exchange factor; IP3: inositol triphosphate; PKA: protein kinase A; PKC: protein kinase C; PLC: phospholipase C.

4. P2X Receptors and Signalling

The family of P2X receptors are non-selective ion channels comprising one or more of seven monomeric proteins (P2X1–P2X7). Each monomeric P2X protein consists of two transmembrane domains (TM1 and TM2) linked via an extracellular ligand-binding loop. The monomeric P2X proteins combine to form trimeric homomultimeric or heteromultimeric ion pores [96–99]; thus, each P2X receptor complex contains three ATP binding sites. At least 13 different trimeric combinations (P2X1, P2X2, P2X3, P2X4, P2X5, P2X7, P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/5, P2X2/6, and P2X4/6) have been reported and functionally characterised in vitro and partly in vivo [28,99]. Of note, P2X6 exists only in heteromeric combinations. Binding of ATP to the extracellular ligand-binding domain induces conformational changes in the multimeric ion pore, leading to opening of the pore and allowing the passage of ions into the cell. P2X receptors are generally known as non-selective cation channels, mainly permeable to Na+, K+, and Ca2+ under physiological
conditions, although a recombinant P2X5 receptor has been shown to allow the passage of Cl\(^{-}\). Excitable cells are thus depolarised upon activation of P2X receptors. Moreover, increased intracellular Ca\(^{2+}\) levels initiate a diverse array of Ca\(^{2+}\)-dependent signalling pathways, both in excitable and non-excitable cells, that regulate various cellular processes, including cell migration, proliferation, necrosis, and apoptosis.

**P2X Receptors and Endothelial Barrier**

P2X receptors are widely expressed throughout the cardiovascular system. mRNA and protein of all P2X receptors have been detected in the endothelium of various types of blood vessels [100–107], but—with the possible exceptions of P2X4 and to some extent P2X7—their roles are unclear [100,103,108]. Human venous endothelium expresses higher levels of P2X4 than arterial endothelium [109]. The most studied human primary ECs are umbilical vein ECs (HUVECs), which express primarily P2X4 and P2X7 and low levels of P2X6 receptors [107] (unpublished data). P2X4 receptors mediate shear stress-induced Ca\(^{2+}\) currents in endothelium [110] that may be responsible for shear stress-mediated endothelial NO production and vasodilation [111]. The vessels from P2X4\(^{-/-}\) mice do not show an EC response to flow, such as calcium influx and subsequent production of NO [112]. A loss-of-function mutation in the human P2X4 receptor is associated with increased pulse pressure [113]. Cardiac ectopic expression of the P2X4 receptor was protective in a mouse model of heart failure [114]. Accordingly, the P2X4 receptor was the major regulator of ischemic preconditioning-mediated neuroprotection [87]. In HUVECs, the P2X4 receptor associates with VE-cadherin and may be involved in the regulation of cell–cell junctions [100]. In this context, we observed that ivermectin, a positive modulator of the P2X4 receptor, attenuated thrombin-induced HUVEC monolayer hyperpermeability (Figure 3). On the other hand, the P2X4 receptor is also reported to be an inflammation-regulated purinergic receptor. In rabbit aortic endothelium, the expression of P2X4 was upregulated after balloon injury followed by a high-fat diet [115]. A high-glucose and palmitate diet induced upregulation of P2X4 and P2X7 receptors accompanied by hyperpermeability of HUVEC monolayers that was attenuated by respective antagonists [116]. In line with this, ATP-mediated coronary microvascular endothelial barrier stabilisation was strengthened in the presence of P2X4 receptor antagonist (5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4 diazepin-2-one (5-BDBD)) and attenuated in the presence of the receptor modulator ivermectin [78]. Differential effects of P2X4 receptor activation on endothelial barrier function under different experimental conditions may be partly explained by the downstream signalling mechanisms. For example, under basal conditions, ECs express high levels of endothelial NO synthase (eNOS), which has been reported to be downregulated under chronic inflammatory conditions that may result in an upregulation of reactive oxygen species production, leading to barrier failure.

Unlike P2X4 receptors, activation of P2X7 receptors in ECs is primarily linked to a proinflammatory and hyperpermeability response. In an in vitro model of the blood–brain barrier, ATP induced an increased production of matrix metalloproteinase 9 (MMP9) in an interleukin (IL)-1\(^{\beta}\)-dependent manner, which was responsible for the degradation of tight junction proteins [117]. These ATP effects were abrogated by P2X7 receptor antagonist, suggesting that they were P2X7 receptor-dependent. Similarly, hyperglycaemia induced the production of IL-1\(^{\beta}\) via P2X7 receptor activation and caused damage to the retinal endothelial cell–cell junctions and barrier that was abrogated by a selective P2X7 receptor antagonist [118]. Likewise, in an in vivo model of intracranial haemorrhage, an upregulation of P2X7 receptor expression accompanied by the development of cranial oedema was observed. Pharmacological inhibition or siRNA-mediated knockdown of P2X7 receptors attenuated the disruption of the blood–brain barrier and the resultant oedema [88]. These effects were mediated via P2X7-induced activation of the RhoA/Rock pathway. Likewise, P2X7\(^{-/-}\) mice were protected against traumatic brain injury-induced development of brain oedema [89] and also the development of lung inflammation and oedema in vivo [119]. In contrast, Kaiser et al. [90] reported a protective role of P2X7
receptors in a cerebral transient IR model of brain injury and oedema formation. The mice deficient in P2X7 receptors developed more severe oedema after transient cerebral artery occlusion compared with their wild-type littermates [90].

Figure 3. Effect of P2X4 receptor modulator (ivermectin; IVM) and antagonist ([5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one: 5-BDBD) on thrombin-induced endothelial hyperpermeability. HUVEC monolayers cultured on filter membranes were exposed to human thrombin (Thr, 0.3 IU/mL) in the absence (red) or presence (green) of ivermectin (IVM; 50 µM) and the flux of labelled albumin was measured as described previously [84]. In a parallel set of experiments, P2X4 receptor antagonist (5-BDBD; 10 µM) was added before the addition of ivermectin and thrombin. n = 4, *p < 0.05 vs. control, #p < 0.05 vs. Thr alone, §p < 0.05 vs. IVM + Thr. For experimental details, please see methods in Supplementary File.

5. P2Y Receptors and Signalling

P2Y receptors are membrane-bound class A GPCRs for extracellular nucleotides [120]. At present, eight mammalian P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) have been cloned and are further classified into two sub-families based on sequence similarities and signal transduction pathways [121–128]. The P2Y1-like subfamily includes the P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors that are coupled to Gq proteins. The P2Y11 receptors are coupled additionally to Gs proteins, activation of which leads to an activation of AC and enhanced production of cAMP [128,129]. The P2Y12-like subfamily includes P2Y12, P2Y13, and P2Y14 receptors, which mediate cellular signalling via Gi proteins [128], activation of which leads to inhibition of AC and reduction in cellular cAMP levels [127,128]. Moreover, activation of several P2Y receptors is associated with activation of the MAPK pathway, and consequently these receptors are involved in cell survival and proliferation [123,130].

P2Y Receptors and Endothelial Barrier

ECs express several of the P2Y receptor subtypes that are distributed over the entire vasculature. The endothelial P2Y receptors have been investigated primarily within the context of their NO-mediated vasorelaxant properties; therefore, fewer data are available in relation to their role in maintaining the endothelial barrier. The P2Y1 receptor is a ubiquitously expressed endothelial purinergic receptor on most EC types. It is a Gq-linked GPCR that has been well-studied in platelet biology, for which ADP acts as a natural agonist and ATP an antagonist [131,132]. P2Y1 knockout mice are viable, fertile, normal in size, and do not present gross physical or behavioural abnormalities [133].
P2Y<sub>1</sub>(−/−) homozygous mice are more susceptible to lung infections and are resistant to ADP/collagen-induced thrombin formation [133, 134]. Moreover, P2Y<sub>1</sub>(−/−) apoE(−/−) double knockout mice have reduced amounts of atherosclerotic lesions [91] that were not affected by transplanting wild-type bone marrow to the knockouts, suggesting the vascular but not the haematopoietic P2Y<sub>1</sub> receptor may be involved in the atherogenic response [91]. Moreover, leukocyte recruitment to inflamed vessels was reduced in vivo and leukocyte transendothelial migration was reduced in P2Y<sub>1</sub> knockout as well as P2Y<sub>1</sub> receptor antagonist-treated ECs in vitro [135]. These studies suggest that the P2Y<sub>1</sub> receptor may potentiate vascular inflammation and hyperpermeability. However, in a mouse model of traumatic brain injury, development of cerebral oedema was ameliorated in mice treated with the P2Y<sub>1</sub> agonist 2-methylthioadenosine 5′diphosphate (2MeSADP). These protective effects of the P2Y<sub>1</sub> agonist were lost in inositol 3-phosphate receptor 2 (IP3R2)-knockout mice, suggesting that it is an IP3/Ca<sup>2+</sup>-dependent phenomenon [92]. We observed that P2Y<sub>1</sub> mRNA is expressed in HUVECs, and treatment of cultured HUVEC monolayers with ADP as well as P2Y<sub>1</sub>-selective agonist 2MeSADP antagonised thrombin-induced HUVEC hyperpermeability (Figure 4). This barrier-protective effect of P2Y<sub>1</sub> agonist is probably mediated via G<sub>q</sub>/IP3/Ca<sup>2+</sup>-dependent activation of Rac1 [136].

**Figure 4.** Effect of ADP and P2Y<sub>1</sub> antagonist (MRS2500) on thrombin-induced endothelial hyperpermeability: HUVEC monolayers cultured on filter membranes were exposed to human thrombin (Thr, 0.3 IU/mL) in the absence (red) or presence (blue) of P2Y<sub>1</sub> receptor agonist ADP (10 µM), and the flux of labelled albumin was measured as described previously [84]. In a parallel set of experiments P2Y<sub>1</sub> receptor antagonist (MRS2500; 10 µM; black) was added before the addition of ADP and thrombin. n = 4, * p < 0.05 vs. control, # p < 0.05 vs. Thr alone, § p < 0.05 vs. ADP + Thr.

P2Y<sub>2</sub> and P2Y<sub>4</sub> are G<sub>q</sub>/G<sub>11</sub>-coupled receptors that are activated by both UTP and ATP [128]. Global deletion of the P2Y<sub>2</sub> gene reduces shear stress-induced vasodilation and hypertension [137]. However, P2Y<sub>2</sub>-knockout mice show reduced inflammatory cell infiltration into injured vessels [138], and endothelial-specific deletion of the P2Y<sub>2</sub> receptor in apoE(−/−) mice results in reduced inflammatory response and increased plaque stability [95], suggesting a pathological role of chronic P2Y<sub>2</sub> receptor activation under inflammatory conditions. Accordingly, knockdown of P2Y<sub>2</sub> receptors in HUVECs ameliorated LPS-induced transendothelial migration of activated neutrophils [139].

P2Y<sub>4</sub>-null mice are viable but display microcardia (small hearts), suggesting that the P2Y<sub>4</sub> receptor plays a role in postnatal heart development [140]. Interestingly, cardiac ECs and not cardiomyocytes express the P2Y<sub>4</sub> receptor, and loss of the P2Y<sub>4</sub> receptor in cardiac
ECs results in reduced growth and migratory capacity in vitro [140]. Surprisingly, P2Y\textsubscript{4} knockout mice are protected from myocardial ischaemic injury, cardiac inflammation, and fibrosis in a left anterior descending (LAD) coronary artery ligation model [94]. Moreover, P2Y\textsubscript{4}-knockout mice are protected from an LPS-induced cardiac microvascular hyperpermeability response. These data suggest that although the endothelial P2Y\textsubscript{4} receptor is required for normal development of the heart in mice, its activation may induce vascular hyperpermeability under pathological conditions.

P2Y\textsubscript{6} is a G\textsubscript{q}-coupled receptor activated by UDP [128] that is expressed on aortic and cerebral ECs [141,142]. Global loss of P2Y\textsubscript{6} receptors results in macrocardia (larger heart), and mice lacking the P2Y\textsubscript{6} receptor show an amplified pathological cardiac hypertrophic response [143]. However, vascular deficiency of P2Y\textsubscript{6} receptors results in reduced vascular inflammation and ameliorated neointima formation in an atherosclerosis mouse model [95,144]. In contrast, inhibition of cerebral P2Y\textsubscript{6} receptors with a selective antagonist aggravates development of cerebral oedema in a mouse model of ischaemic brain injury [145].

P2Y\textsubscript{11} is the only known human P2Y receptor coupled to G\textsubscript{s} [124,128,129]. The murine orthologue of the P2Y\textsubscript{11} receptor does not exist or at least has not yet been identified. Moreover, the lack of selective agonists and antagonists for this receptor as well as specific detection tools (antibodies) make functional investigations of the P2Y\textsubscript{11} receptor difficult [146]. We did not detect P2Y\textsubscript{11} mRNA in HUVECs, but other EC types were not investigated. Presumably, if it is expressed in some EC type, one would expect its activation would raise intracellular cAMP levels that can interact with multiple signalling pathways, e.g., Rac1-dependent actin cytoskeleton rearrangement and MLCP-mediated inactivation of the contractile machinery, thus modulating endothelial barrier properties.

The P2Y\textsubscript{12}-like subfamily comprises three members: P2Y\textsubscript{12}, P2Y\textsubscript{13}, and P2Y\textsubscript{14}. All of these receptors are coupled to G\textsubscript{i}, and their activation leads to suppression of AC activity and cAMP production [128]. P2Y\textsubscript{12} is well-studied in relation to platelet biology, and its antagonists are used clinically as anticoagulants in various pathological conditions. In human cardiac-derived mesenchymal cells, ticagrelor, a P2Y\textsubscript{12} receptor antagonist, induces the release of anti-apoptotic exosomes [147] that may also modulate the coronary microvascular endothelial barrier. Endothelial expression of both P2Y\textsubscript{12} [84,148] and P2Y\textsubscript{13} [104] has been documented. Recently, we demonstrated the expression of P2Y\textsubscript{12} receptor mRNA and protein in HUVECs, and a specific P2Y\textsubscript{12} antagonist increased intracellular cAMP levels and protected against thrombin-induced hyperpermeability [84]. We also observed the expression of P2Y\textsubscript{13} but not P2Y\textsubscript{14} mRNA in primary HUVECs (unpublished). In vasa vasorum ECs, ADP mediates a mitogenic response partly via P2Y\textsubscript{13} receptors [104]. The expression of P2Y\textsubscript{14} receptor has been reported in rat primary brain microvascular ECs [149], and activation of this receptor induces a pro-inflammatory response in ECs. Moreover, UDP-glucose (an agonist for P2Y\textsubscript{14} receptor) mediated a contractile response in isolated pancreatic arteries in an endothelium-dependent manner, and this effect was abrogated by a selective P2Y\textsubscript{14} receptor antagonist [150]. No further data are available related to the involvement of P2Y\textsubscript{13} and P2Y\textsubscript{14} receptors in the control of endothelial barrier properties. Figure 5 presents an overview about the effects of various P2Y receptors’ activation on endothelial barrier function.
6. Conclusions and Perspective

Endothelial barrier properties are influenced by extracellular nucleotides via activation of various purinergic receptors. The response depends on the type of receptor(s) present and the local concentration of the nucleotides. Adenosine, primarily via activation of A2A and A2B receptors, raises intracellular levels of cAMP in the lung microvascular bed and thus strengthens the barrier properties and ameliorates hypoxia- and inflammation-induced development of oedema. Selective agonists for adenosine A2 receptors are available that may be tested (for local application) for clinical use in various oedematous abnormalities of the lung, e.g., acute lung injury. Conversely, A2 receptor activation in brain and coronary microvasculature results in transient opening of the cell–cell junction in a cAMP-dependent manner. This property of the brain microvasculature can be exploited for local delivery of drugs to the CNS. P2 receptors are also widely distributed in the vascular bed. Chronic P2X receptor activation leads to endothelial barrier destabilisation and oedema formation, an effect primarily attributed to the P2X7 receptors. There is a need for the development of more selective and potent P2X7 receptor antagonists to ameliorate inflammation-induced loss of endothelial barrier function. There is also a lack of selective P2Y receptor agonists and antagonists, which makes the investigation of P2Y receptors in relation to endothelial barrier function difficult. We and others have documented that ATP at low micromolar concentrations stabilises endothelial barrier function, mainly via activation of various P2Y receptors, whereas at high concentration (in the millimolar range), it may act as a danger-associated molecular pattern (DAMP) [151], amplifying the inflammatory response. Inhibition of the P2Y12 receptor blocks inflammation-induced increases in endothelial...
permeability [84]. Further studies are needed to identify specific P2Y receptors that mediate endothelial barrier stabilisation and destabilisation.

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**Abbreviations**

| AJ  | Adherens junctions                        |
| ADP | Adenosine 5′-diphosphate                  |
| AMP | Adenosine 5′-monophosphate                |
| ATP | Adenosine 5′-triphosphate                |
| cAMP | 3′, 5′-cyclic adenosine monophosphate |
| DAMP | Danger-associated molecular pattern |
| DPCPX | 8-Cyclopentyl-1,3-dipropylxanthine |
| ER  | Endoplasmic reticulum                    |
| ERK | Extracellular signal-regulated kinase    |
| GPCR | G protein-coupled receptor               |
| H/R | Hypoxia-reoxygenation                    |
| HUVEC | Human umbilical vein endothelial cells |
| I/R | Ischaemia reperfusion                    |
| LPS | Lipopolysaccharide                        |
| MAPK | Mitogen-activated protein kinase         |
| MEK | MAPK/ERK kinase 1                         |
| MLCK | Myosin light-chain kinase                |
| MLCP | Myosin light-chain phosphatase           |
| MMP9 | matrix metalloproteinase 9               |
| MYPT1 | Myosin phosphatase targeting subunit 1  |
| NECA | 5′-N-Ethylcarboxamidoadenosine            |
| PKC | Protein kinase C                         |
| ROCK | Rho-associated kinase                    |
| ROS | Reactive oxygen species                  |
| UDP | Uridine diphosphate                       |
| UTP | Uridine triphosphate                     |
| XAC | Xanthine amine congener                  |
| VE  | Vascular endothelium                     |
| vWF | von Willebrand Factor                    |
| ZO-1 | Zonula occludens-1                       |
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