Role of purinergic signaling in pathological pulmonary vascular remodeling

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ACADEMIC DISSERTATION

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Science is organized knowledge. Wisdom is organized life.
(Immanuel Kant)

ABSTRACT

Irreversible vascular remodeling has a central role in a variety of pathophysiological conditions including pulmonary arterial hypertension (PAH). Hypoxia and inflammation are prominent features in PAH, along with hyperplasia and hypertrophy of vessel wall layers. Although, endothelial cell (EC) dysfunction is thought to drive the multiple forms of vascular remodeling, the origins of this phenomenon are poorly understood. Extracellular ATP and its metabolites are important regulators of vascular tone, permeability, and homeostasis. Yet little is known about their role in pathological vascular remodeling.

By using chronic hypoxia and PAH animal models as well as human PAH patient samples, this study was undertaken to evaluate the catalytic activities and expression levels of nucleoside triphosphate diphosphohydrolase-1 (NTPDase1, otherwise known as CD39) and other purine-converting ectoenzymes with a primary focus on vascular EC. For this purpose we employed thin-layer chromatographic enzyme assays with ^{3}H-labelled nucleotide substrates, in combination with various immunoassays and qPCR. In addition we have developed a highly sensitive assay for simultaneous sensing of extracellular ATP and its metabolites and also a novel method for measuring CD39 activity in modeled to *in vivo* conditions. In functional assays, cells or animals were stimulated through purine signaling pathways and proliferation, apoptosis, permeability, and DNA damage were assayed.

Our results clearly demonstrated that the activity of CD39 was downregulated in chronic hypoxia, monocrotaline induced animal models of PAH and in human PAH patients. Attenuated enzyme activities could create a niche in the vasculature where ATP levels were increased and adenosine levels were decreased. Even a small increase in ATP concentration was enough to induce an apoptosis-resistant, hyper-proliferative, and DNA-damage-resistant phenotype in ECs of pulmonary origin. The observed effects were at least partly dependent on P2Y11 receptor activation. In addition, we found that low ATP concentrations could induce pulmonary smooth muscle cell proliferation and migration. Interestingly, we found that small apelin peptide could directly restore the downregulated CD39 activity.

This study implies that purinergic signaling, ATP mediated cell activation in particular, plays a truly significant role in pathological vascular remodeling, and that it could be used as a therapeutic target. Moreover, purinergic signaling pathways could be used before vascular injury to precondition EC against irradiation or chemotherapy induced DNA damage.

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LIST OF ORIGINAL PUBLICATIONS

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I. Yegutkin GG, **Helenius M**, Kaczmarek E, Burns N, Jalkanen S, Stenmark K, Gerasimovskaya EV (2011). Chronic hypoxia impairs extracellular nucleotide metabolism and barrier function in pulmonary artery vasa vasorum endothelial cells. Angiogenesis 14(4): 503−513.

II. **Helenius MH**, Vattulainen S, Orcholski M, Aho J, Komulainen A, Taimen P, Wang L, de Jesus Perez VA, Koskenvuo JW, Alastalo TP (2015). Suppression of endothelial CD39/ENTPD1 is associated with pulmonary vascular remodeling in pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol. 2015 May 15; **308**(10):L1046-57.

III. Aho J, **Helenius M**, Vattulainen S, Alastalo T-P, Koskenvuo JW (2015). Extracellular ATP protects endothelial cells against DNA damage. (Submitted).

IV. **Helenius M**, Jalkanen S, Yegutkin GG (2012). Enzyme-coupled assays for simultaneous detection of nanomolar ATP, ADP, AMP, adenosine, inosine and pyrophosphate concentrations in extracellular fluids. Biochim Biophys Acta 1823(10): 1967−1975.

PUBLICATIONS NOT INCLUDED FOR THESIS WORK

Vattulainen S, Aho J, Oreholski M, Rojas V, Yuan K, **Helenius M**, Taimen P, Myllykangas S, De Jesus Perez V, Koskenvuo JW, Alastalo T-P (2014). Loss-of BMPR2 is associated with abnormal DNA repair in pulmonary arterial hypertension. Am J Respir Cell Mol Biol 50(6): 1118−1128.

Alastalo TP, West G, Li SP, Keinänen A, **Helenius M**, Tyni T, Lapatto R, Turanlahti M, Heikkilä P, Kääriäinen H, Laakso M, Mauermann M, Herrmann H, Pihkala J, Taimen P. (2015). LMNA Mutation c.917T>G (p.L306R) Leads to Deleterious Hyper-assembly of Lamin A/C and Associates with Severe Right Ventricular Cardiomyopathy and Premature Aging. Hum Mutat. 2015 Jul; **36**(7):694-703].

ABBREVIATIONS

5'NT	5'-nucleotidase
ADA	adenosine deaminase
ADP	adenosine diphosphate
AK	adenylate kinase
AMP	adenosine monophosphate
AMPC	α,β-methylene adenosine diphosphate
Ang	angiotensin
Ap_5A	diadenosine pentaphosphate
ATP	adenosine triphosphate
BMPR	bone morphogenic protein receptor
BSA	bovine serum albumin
BSS	basal salt solution
cAMP	cyclic AMP
CPCA	5'(N-cycloprolyl)-carboxamido-adenosine
DMEM	Dulbecco´s modified Eagle-Medium
DMSO	dimethyl sulfoxide
EC	endothelial cell
ECM	extracellular matrix
E-NPP	ecto-nucleotide pyrophosphatase
E-NTPDase	ecto-nucleoside triphosphate diphospho-hydrolase
EPC	endothelial progenitor cell
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
HDMEC	human dermal microvascular endothelial cell
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
(i)PAH	(idiopathic) pulmonary arterial hypertension
IHC	immunohistochemistry
IL	interleukin
kDa	kilodalton
MAPK	mitogen-activated protein kinase
MCT	monocrotaline
MVEC	microvascular endothelial cell
Nf-κB	nuclear factor kappa B
KRPG	Kreb´s ringer phosphate glucose
PAP	prostatic acid phosphatase
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PHD	prolyl hydroxylase
POM-1	sodium metatungstate-1
(P)SMC	(pulmonary) smooth muscle cell
TBS	tris buffered saline
TLC	thin layer chromatography
TNF-α	tumor necrosis factor alpha
UTP	uridine triphosphate
VEGF(R)	vascular endothelial growth factor (receptor)
VVEC	vasa vasorum endothelial cell

REVIEW OF THE LITERATURE

1 PURINERGIC SIGNALING

Nucleotides and nucleosides are well recognized as building blocks for DNA and RNA as well as for their chemical energy transfer capabilities. Nucleotides such as adenosine triphosphate (ATP), which conserve the energy obtained from nutrients, are used to drive various energy-dependent reactions inside cells. Nucleotides consist of a purine or pyrimidine base, a five-carbon sugar part (ribose or deoxiribose), and one or more phosphate groups.

Interestingly, purine and pyrimidine compounds are likely to be the most primitive and widespread signaling system found in the Animal, Plant, Bacteria, and Fungi kingdoms (1). In early life forms, it was efficient to use the same molecules for securing genomic data, for energy storage, and for cell signaling. It might be counterintuitive as to why animal cells would release an important substance such as ATP, but the released extracellular ATP concentrations are minute compared to intracellular ones (2). In addition to intracellular ATP-driven signaling and homeostasis, cells are also regulated through extracellular purinergic signaling pathways, which are linked to the intracellular signaling pathways. At present, ATP-mediated cell signaling has been identified in nearly all human tissues and cell types (1). There are three main constituents that together form the extracellular purinergic signaling system (Figure 1): **1**) release of ATP and other purines to the extracellular space; **2**) receptor binding and signal transduction, and; **3**) signal inactivation. Each of these key points will be described below in more detail.

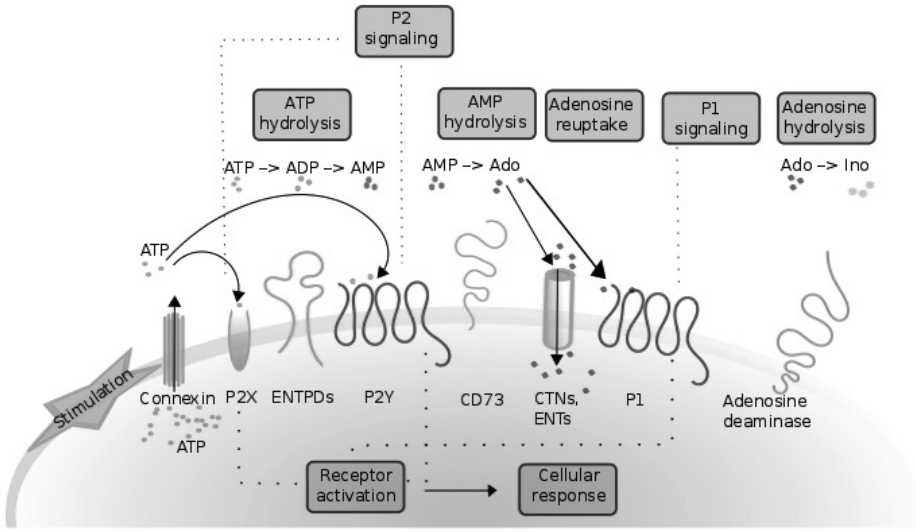

Figure 1. Schematic representation of purinergic signaling. Cells release ATP under various conditions, such as hypoxia and shear stress, but also at certain basal rates through connexin and pannexin channels, exocytosis, and facilitated diffusion. ATP is also released in greater quantities from apoptotic or necrotic cells. Outside the cell, ATP can bind the members of cation gated P2X receptor family or to the G-protein coupled P2Y receptor family, different cell types express these receptors differentially so the response to ATP activation will also vary from one cell type to another. At the same time ATP is readily hydrolysed to adenosine or further through a sequence of cell membrane bound ecto-enzymes, such as CD39 and CD73, as well as by the soluble forms of these enzymes. Adenosine binds to G-protein coupled receptors (P1). In addition, adenosine is readily takenup to the cells through specific transporters, or converted further to inosine and hypoxanthine by additional ecto-enzymes such as adenosine deaminase (ADA) and purine nucleoside phosphorylase.

1.1 PURINE RELEASE TO EXTRACELLULAR SPACE

It took a few decades before the very disputed concept of purinergic signaling was generally accepted. The most controversial question to overcome was: why would cells release ATP to their surrounding and thus waist their valuable source of energy. This apparent contradiction could be explained by the major differences between intra- and extracellular ATP levels. With mitochondrial activity, cells are able to keep the intracellular ATP concentration at a millimolar level (1–10 mM) in normal conditions (3) while extracellular ATP is kept in the low nanomolar range in basal conditions (4, 5) or at sub-micromolar or low micromolar levels in the “ATP-halo” in the close proximity of extracellular membrane (Figure 2) (6).

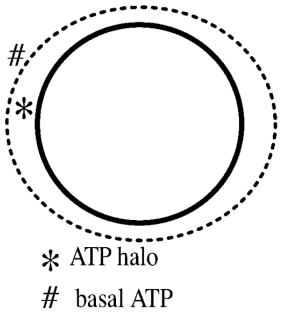

Figure 2. The black circle represents the outer cell membrane of a living cell. It has been proposed that cells can maintain a micromolar ATP concentration in close proximity of the plasma layer. This "ATP-halo", marked with*, can flow to the bulk extracellular ATP pool # where ATP concentration is kept in the nanomolar range in basal conditions.

Non-lytic nucleotide release has been described in nearly all cell types in humans (7). In blood vessels, ATP can be released from vascular smooth muscle cells (8), fibroblasts (9), ECs (10), perivascular sympathetic nerves (11), circulating red blood cells (12), immune cells (13, 14), and platelets (15). Released ATP elicits its effects in an autocrine or paracrine manner while the amount of freely circulating ATP in the blood stream is usually maintained within the low nanomolar range (16). There are three main routes that cells use for ATP release: membrane ion channels, facilitated diffusion, and exocytotic secretion (2).

Sympathetic nerve cells that release noradrenaline use ATP as a co-transmittor through vesicular exocytosis (17) to produce a junction potential at the smooth muscle cells, which will lead to vasoconstriction. ECs have been shown to release ATP through vesicular exocytosis in response to changes in vessel blood flow and shear stress (18). It was unclear for a long time how ATP could be packed to these excitatory vesicles until vesicular nucleotide transporter (VNUT) that pumps ATP into the vesicle lumen was characterized (19). Yet, VNUT remains uncharacterized in perivascular nerve cells and ECs.

Besides vesicular exocytosis, it is possible that ECs along with some other vascular cells use ATP binding cassette (ABC) transporters for facilitated diffusion of ATP. The ABC transporters use ATP as an energy source for facilitated diffusion across the cell membrane. Their role in direct ATP release remains controversial, but these transporters have been characterized in vascular smooth muscle cells (20), ECs (21), red blood cells (22), and platelets (23).

Electrodiffusional movement through gap-junction protein (connexin and pannexin) hemichannels has been characterized in the majority of different cell types (24). On the cell surface, connexins (Cx) form multimers called connexons, which bind to the connexons of neighboring cells, connecting the cytosols of these two cells. Altogether 20 mammalian connexin isoforms have been characterized however, these connexons have been reported

to also exist as independent units on the cell surface of various cell types (24) including vascular ECs (25), vascular smooth muscle cells (26), and fibroblasts (27). It is hypothesized that these unconnected connexin channels, could release ATP to the extracellular milieu after stimulation such as a decrease in the extracellular Ca^{2+} concentration(28), membrane depolarization (29) or mechanical activation (30). In the vascular wall, especially Cx37, Cx40, Cx43, and Cx45 isoforms are expressed (31).

Pannexins (Panx) are a recently discovered protein family that consists of three isoforms. Panx1, Panx2, and Panx3 have been identified in numerous mammalian cell types (24). Pannexins are orthologs of the invertebrate gap junction proteins and their membrane topology is similar to connexins (32). However, pannexins are unable to form gap junctions in mammalian cells (33). In the context of the vasculature pannexin, channel-mediated ATP release has been identified in the following cells: T-cells after inflammation (34), smooth muscle cells after α1D adrenergic receptor activation (35), endothelial cells after thrombin stimulation (36), and erythrocytes in hypoxic conditions (37). The ATP release through pannexin channels has been shown to be calcium insensitive (38). It has not been clearly shown which cell type is responsible for the majority of ATP release in the vasculature. Though, certainly the ECs have the most versatile machinery, as they are known to use all the previously mentioned routes for ATP release.

1.2 PURINE MEDIATED CELL SIGNALING

Cyrus Fiske and Yellapragada Subbarow discovered ATP independently in 1929. Already during the same year Drury and Szent-Györgyi described ATP's potent actions on several cell types (39). Still, only in 1976 Geoffrey Burnstock defined the first purine receptors (40). Currently, two purinoreceptor groups have been identified – namely P1 and P2 (41).

The P1 receptors are adenosine receptors, also called ADORA in the literature. Currently, four different members of the P1 receptor family have been characterized (A_1, A_{2A}, A_{2B}, and A_3), all of which are G-protein-coupled receptors (42). In addition, these receptors can form homo- or heterodimers even with other receptor types (43-45). The P1 adenosine receptors are mainly involved in inhibition (A_1 and A_3) or activation (A_{2A} and A_{2B}) of adenylate cyclase (41).

The ionotropic $P2X_{1-7}$ trans-membrane receptors are only activated by ATP or ATP-like agonists (41). The P2X receptor monomers form pore-like oligomeric structures on the cell surface, which allow cation movement across the cell membrane. The formed homo- or hetero-oligomers are usually trimers or hexamers. (41, 46). Besides hetero-oligomerization, a huge variety of forms and functions are created through P2X receptor splice variants (47). Activation of a functional P2X receptor causes a conformational change in the receptor structure, which leads to formation of a small pore permeable to Na^+ K^+, Mn^{2+}, Mg^{2+}, and Ca^{2+} causing depolarization and activation of various Ca^{2+} sensitive pathways (48).

There are eight functional human P2Y receptors identified: $P2Y_{1–4}$, $P2Y_6$, and $P2Y_{11, 12, 14}$ (49). In contrast with P2X receptors, the genes coding for P2Y receptors lack introns (except for $P2Y_{11}$). This means that there are fewer splice variants in the P2Y receptor family (50).

Several secondary messengers are formed upon G-protein coupled P2Y receptor activation. $P2Y_{1,2,4,6}$ receptor activation will increase intracellular inositol triphosphate (IP_3) and calcium concentrations. The activation of $P2Y_{11}$ receptor can lead to increase in cAMP, IP_3, and calcium levels. The activation of most P2Y receptors initiates several mitogen-activated protein kinase pathways (MAPKs), especially extracellular signal-regulated protein kinase 1/2 (ERK 1/2)(50).

P2 receptors show different agonist specificity, which covers purine and pyrimidine nucleotides in a much broader spectrum compared to the P2X receptor family. The $P2Y_{1,2,11,12}$ receptors bind to ATP, ADP or both; $P2Y_{2,4,6}$ receptors bind to UTP and UDP; and $P2Y_{14}$ receptor binds to UDP-glucose and UDP-galactose (41, 50). In addition, synthetic agonists or antagonists for most of these receptors are known (41). Also, the G-protein coupled receptors can form dimers but they can also form couples with non-P2Y receptors. For example, the $P2Y_1$ receptor can form homodimers (51), hetero-oligomers with $P2Y_{11}$ receptors (52), but also with adenosine A_1 receptors (53). In addition, it is evident that the P2Y receptors have synergistic crosstalk between the P2X receptor family members (54, 55).

Taken together, numerous purine receptors with complicated interactions are known. In practice, naming only one receptor that is responsible for some ATP mediated effect is very likely to be a simplified model. More accurately, the cell´s response to ATP mediated signaling is a net result of the variety of purine receptors, expressed by the cell type of interest. Expression patterns of purine receptors vary greatly between organisms, cell types, and even between the same cell types in different tissues (41, 56-58). An expression analysis study with human umbilical vein endothelial cells (HUVEC) and smooth muscle cells (SMC) from mammary artery suggests that $P2X_1$, $P2Y_2$, and $P2Y_6$ are the most abundantly expressed P2 receptors in SMCs and the $P2X_4$, $P2Y_{11}$, $P2Y_1$, and $P2Y_2$ are the most abundantly expressed P2 receptors in ECs (59).

1.3 ECTO-ENZYMATIC REGULATION OF PURINERGIC SIGNALING

Ectonucleotidases and kinases are directly linked to P1 and P2 receptor signaling at multiple levels. Firstly, these ecto-enzymes are needed for termination of receptor activation and to avoid receptor desensitization, and secondly, for generation of P1 and P2 receptor ligands from available substrates. In addition, some of the enzymes such as NTPDase1/CD39 are expressed in very close proximity to a number of P1 and P2 receptors where they could directly control receptor activation and prevent desensitization (60, 61).

1.3.1 The NTPDase family

There are eight members in the nucleoside triphosphate diphosphohydrolase (NTPDase) protein family. However, many additional names for these enzymes have been used in the literature previously, such as ATPase, apyrase, ATP-diphosphohydrolase, and nucleoside diphosphohydrolase. Four NTPDase family members are expressed at the cell surface (NTPDase1, 2, 3, and 8); two are intracellular enzymes (NTPDase 4 and 7) and two are secreted enzymes (NTPDase 5 and 6) (62). Maximal NTPDase activity is reached at pH values between 7–8.5 and millimolar concentrations of Mg^{2+} or Ca^{2+} cations as co-factors. The NTPDase substrate specificity is towards different purine and pyrimidine nucleoside tri- and di-phosphates, including biologically active ATP, UTP, ADP, and UDP with apparent K_m values between 50–200 μM (63, 64). The cell membrane expressed NTPDases are highly glycosylated proteins (70-80 kDa) with two trans-membrane domains (64). On the cell surface NTPDases exist as monomers or as homo-oligomeric structures with increased catalytic activity (65).

Of the eight NTPDases, NTPDase1/CD39 is the best-characterized enzyme that is highly expressed in the vasculature by ECs, SMCs, leukocytes, but also in other organs and cell types (64, 66). While NTPDase1 is mainly expressed on the luminal side of vessels, the NTPDase2 is highly present in pericytes and advential cells in larger muscularized vessels (67, 68).

1.3.2 The nucleotide pyrophosphatase/phosphodiesterase (NPP) family

There are seven iso-enzymes in the NPP-family (NPP1-NPP7) that are capable of hydrolyzing pyrophosphate and phosphodiester bonds in nucleotides, diadenosine polyphosphates, nucleic acids, choline phosphate esters, and lysophospholipids (2, 64). The cell surface transmembrane glycoprotein NPP enzymes 1 and 3 are capable of hydrolyzing ATP and other nucleotides (69). These enzymes can also form dimers or exist in soluble form (62, 70). The apparent K_m values for ATP hydrolysis by NPP1 and NPP3 are in the 100–300 μM range, much like NTPDase1 (62, 64). This makes the NPP family enzymes very relevant for the purinergic signaling cascade, although these enzymes are often severely neglected over the NTPDase1. However, the role of NPP enzyme catalyzed ATP hydrolysis on endothelium is less important than NTPDase1 activity (71).

NPP1 is highly expressed in bone, cartilage, heart, liver, placental, kidney, and testis tissues, where NPP1 down-regulation may lead to tissue and vessel calcifications (64, 72). NPP3 is mainly expressed at the apical membranes of hepatocytes and cholangiocytes but it is also co-expressed with NPP1 in many other epithelial surfaces (62, 69). In the context of vascular purine homeostasis, a particular attention is given to soluble forms of NPP enzymes. In particular, NPP1 that is found in human serum, is an important regulator of vascular purine homeostasis (73).

1.3.3 Ecto-5'-nucleotidase/CD73

Ecto-5´-nucleotidase (otherwise known as CD73) is a glycol phosphatidylinositol (GPI) anchored glycosylated ecto-enzyme that catalyzes the hydrolysis of AMP to adenosine (K_m 10–40 μM)(74, 75). CD73 is abundantly expressed by vascular ECs, but also by leukocytes and other cells (75-77). In addition, the soluble form of ecto-5'-nucleotide is found in the serum (78). CD73 has been extensively studied in different contexts (64) and interestingly it has been linked to many vascular diseases such as neointima formation (79) vascular inflammation (80) and cardiac allograft vasculopathy (81).

1.3.4 Alkaline phosphatase (AP) family

These homo- or heteromeric GPI-anchored plasma membrane enzymes (~80 kDa) are ubiquitously expressed in mammals through four genes: ALPL, ALPP, ALPP2, and ALP1. APs need Zn^{2+}, Mg^{2+} and alkaline conditions (pH 8–11) for catalytic activity. Their substrate range is very broad including various phosphomonoesthers, nucleotides, and many other phosphate-containing compounds (62, 64). With the exception of blood-brain barrier ECs, resting vascular cells do not express high amounts of APs. AP expression can be induced by cytokine IL-6 in ECs (82, 83) and by tumor necrosis factor alpha (TNF-alpha) in vascular SMCs (84). Several circulating vascular cells such as erythrocytes (85) and leukocytes (86) (87) express significant amounts of APs. In addition, soluble APs are found in serum (88).

1.3.5 Acid phosphatases (PAP, TRAP)

There are two isoforms of transmembrane prostatic acidic phosphatases (PAP). These enzymes can hydrolyse AMP and thiamine monophosphate (TMP) at pH 4–8 (89). Another type of acidic phosphatase is the tartrate-resistant acid phosphatase (TRAP), which can hydrolyze several phospho-compounds including adenine nucleotides at an acidic pH (90). In the vasculature, acid phosphatases are mainly expressed by some of the circulating cells, such as macrophages and dendritic cells, but soluble forms also exist (64, 90).

1.3.6 Adenylate kinase (AK)

Adenylate kinases catalyze ADP formation from ATP and AMP through reversible phosphotransfer reactions (ATP + AMP → 2 ADP). There are numerous intracellular isoforms of AKs but AK1 and AK1-beta are the best-characterized membrane- bound ectoenzymes (64). Both soluble and membrane-bound AKs are present in vascular ECs (91) and lymphocytes (92), as well as serum (93).

1.3.7 Nucleoside diphosphate kinase family (NDPK)

These oligomeric proteins are formed by 17-20 kDa subunits and in adenosine nucleotide metabolism they catalyze the formation of ATP from ADP and other nucleoside triphosphates (ADP+NTP→ATP) (64). The eight non-metastatic cell (NME) genes (also

known as nonmetastatic-23 (NM23)) encode the NDPK family proteins that are expressed as membrane-bound enzymes on vascular endothelial, smooth muscle cells, and lymphocytes (10, 76, 91, 92). Soluble NDPK forms also exist in serum (93).

1.3.8 ATP synthase

It has recently been shown that endothelial cells, lymphocytes, and some other cell types, could express an active form of ecto-ATP-synthase on the outer face of their plasma membrane (64, 94). It has been reported that this ATP synthase could play a role in angiogenesis and immune responses (95, 96). Nevertheless, it remains an open question as to whether ATP-synthase, AK or NDPK are responsible for the observed ATP production. Altogether, the highly versatile network of ATP releasing mechanisms and ecto-enzymes, together with the vast repertoire of receptors, provides great plasticity for appropriate cell responses in different tissues and cell types.

2 Vascular biology

The cardiovascular system consists of the heart, lungs, arteries, veins and blood (Figure 3), and is essentially supported by the lymphatic system, which includes lymph nodes, lymphocytes, tonsils, spleen, thymus, lymphatic vessels, and lymph. The cardiovascular system provides oxygen and fresh nutrients for the whole organism, whilst removing CO_2 and other harmful metabolites from tissues. Blood also transports immune cells, hormones, other messenger molecules and drugs throughout the body and it transmits heat from the core to the extremities. The cardiovascular system has a central role in wound healing, tissue regeneration, and re-organization.

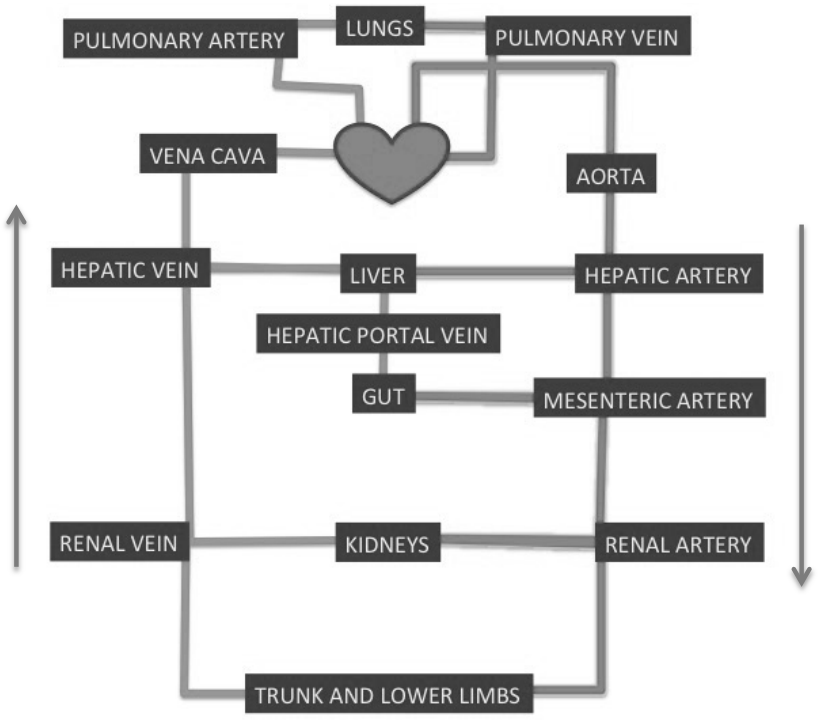

Figure 3. Schematic presentation of the human cardiovascular system. Blood flows from the left ventricle to the aorta, which supplies the gut, kidneys, trunk, and lower limbs. All of the veins collect deoxygenated blood and ultimately lead to the right atrium through the superior or inferior vena cava. The deoxygenated blood is pumped from the right atrium to the right ventricle and from there to the lungs to collect more oxygen. Oxygenated blood is collected to the pulmonary vein, which leads to the left atrium. From the left atrium the oxygenated blood is pumped to the left ventricle and again to the systemic circulation.

2.1 BLOOD VESSEL FORMATION

The vascular network differentiates from blood islets of the splanchnopleuric mesoderm. Mesoderm is the middle layer in the developing embryo between the ectoderm and endoderm. Blood vessels depend on the EC lining that can be tailored to fit local requirements. Remodeling of the endothelial cell network makes tissue growth and repair possible. Large arteries consist of a wall of connective tissue and smooth muscle cell layers, which are lined by a single-cell thick endothelial cell layer with basal lamina in between. Smaller arteries lack surrounding smooth muscle cells, but a sparse network of pericytes stabilizes the vessels (97). However, even large veins lack thick smooth muscle cell layer. During embryonic development the main vessels, such as the aorta, form in situ by proliferation and differentiation from previously avascular tissue. This process is called vasculogenesis and it is derived from endothelial progenitor cells (98). The early blood-vessel network is then finalized with active angiogenesis (angiogenic sprouting). Angiogenesis or neovascularization means the formation of new blood vessels from the existing ones (99). The newly-

formed vessels mature by integrating endothelial cells to the surrounding cells and matrix. In adults, angiogenic vessel growth is required for example during wound healing and in the female reproductive cycle (99). New endothelial cells for angiogenesis can arise not only through mitotic cell divisions, but also in post-natal individuals from endothelial progenitor cells (EPC), mesoangioblasts, and multipotent progenitor cells or from side-population cells in the bone marrow (100, 101).

Five clear steps (Figure 4) can be identified during the angiogenic process: **1**) pericyte detachment from the ECs; **2**) degradation of basal lamina and extracellular matrix by proteases such as matrix metalloproteinases, chymases and heparinases; **3**) migration and proliferation of endothelial cells; **4**) formation and fusion of newly formed vessels; and **5**) blood flow (99, 102). Vascular ECs are rather stable and stationary under normal conditions, however in angiogenesis they need to actively proliferate and migrate (99). The purpose of this tightly regulated process is to provide oxygen and glucose where it is needed more and to provide a channel for cells to dispose their harmful waste material(99). Thus, physiological conditions such as hypoxia and hypoglycemia can trigger the formation of new blood vessels from the existing ones. However, dysregulated angiogenesis has a major role in various diseases, such as cancer, asthma, atherosclerosis, hypertension, pre-eclampsia, and diabetes (103).

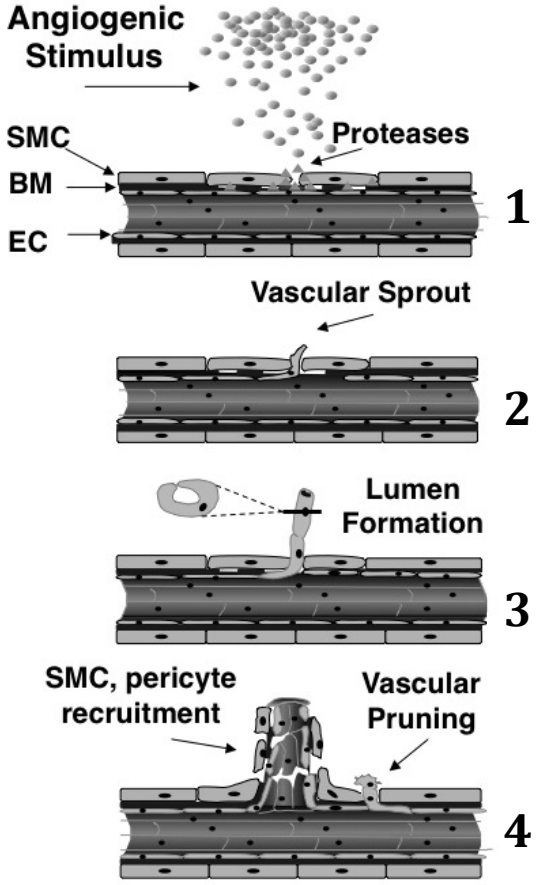

Figure 4. Schematic representation of angiogenesis. 1. The need for new vasculature triggers the release of angiogenic factors such as VEGF, which leads to EC activation and secretion of proteases. ECs break free of cell-cell junctions and ECM attachments and the basal lamina membrane (BM) is disrupted. 2. Tip cells start to migrate towards chemoattractants and vascular sprouts are formed. 3. When two tip cells come together, ECs circularize and create a lumen allowing blood flow. 4. Newly formed vessels mature and stabilize after SMC and pericyte recruitment. However, not all sprouts lead to mature vessels but the unnecessary ones are pruned away.

The most important growth factor, which controls new vessel formation in vasculogenesis and angiogenesis, is vascular endothelial growth factor (VEGF) (98). In addition several other factors such as angiopoietins and ephrins are needed to complete vascular remodeling and maturation (98). In the vast VEGF family, there are members with structural homologies to platelet derived growth factors (PDGF), four of which are the main VEGF prototypes (A, B, C, and D) (99, 104). The three main tyrosine kinase receptors, which recognize VEGF family members, are VEGFR-1 (also known as Flt-1), VEGFR-2 (also known as KDR or Flk-1), and VEGFR-3 (also known as Flt-3) (98). Out of these receptors, VEGFR-2 is the major mediator of growth and permeability, VEGFR-1 has an inhibitory role sup-

pressing the effects of VEGFR-2 activation and VEGFR-3 may be important for vessel development (98). In adults, epithelial cells close to fenestrated endothelial cells express VEGF, which emphasizes its other important role in controlling vessel permeability (105). In addition, VEGF expression by endothelial cells is upregulated by hypoxia, hormones, hypoglycemia, and advanced glycation end products (99). VEGF signaling activates several intacellular secondary messanger cascades through extracellular kinase 1/2 (ERK 1/2), phosphatidylinositol-3 kinase (PI3K), protein kinase C, and phospholipase Cγ activation, which are all involved in vascular function control (106). VEGFR-2 inhibitors are used to block tumor angiogenesis in cancer threapies. However, these treatments can induce severe vascular and cardiac toxicity by increasing blood pressure and compensatory hypertrophy inhibition (107, 108).

Angiopoietin signaling through Tie-2 tyrosine kinase receptors complements the VEGF system and is needed especially later during maturation, branching and organization of vessels (109). There are four isoforms of angiopoietins (1–4), of which Ang-1 and Ang-2 are the most characterized (98). Ang-1 signaling through Tie-2 receptors on ECs leads to the recruitment of auxiliary cells and the production of extracellular matrix (ECM) through transforming growth factor beta (TGF-β) activation, while Ang-2 transmits the opposite effects (99). Ang-1 is a VEGF counter effector, which decreases vascular permeability and stabilizes the newly formed vessels (110, 111).

The largest growth factor receptor family is the ephrin tyrosine-kinase-receptor family (98). Ephrin-B2 and its receptor EphB4 both have important roles in vascular development (112). Interestingly, ephrin-B2 is only expressed in arteries and EphB4 in veins, which creates the first tool for molecular distinction between arteries and veins (112). This polarity also suggests that ephrin-B2 and Eph4B are needed for the maturation of a new vessel into an artery or a vein (99). In addition to these, several EC growth factors and a number of cell-adhesion molecules such as integrins are essential in angiogenesis regulation (110, 113).

Angiogenesis occurs through capillary sprouting where a tip cell forms many long filopodia and stalk cells follow (97). The tip cell invades towards a VEGF gradient (114). Hypoxia and VEGF both induce Notch receptor ligand Delta-like 4 (Dll4) expression in vessel sprout tip cells. Tip cells then signal through Notch 1 to the adjacent cells, turning them to stalk cells (115). This is because new vessels need to grow in specific directions. Notch signaling inhibits VEGF receptor expression in stalk cells (116). At the end stage of angiogenesis, the stalk cells form tubular structures where oxygenated blood starts to flow, which leads to VEGF down regulation that allows proper lumen formation and vessel maturation (117). The angiogenesis process ends only after the tip cell encounters another tip cell or a vessel to complete the blood circulation circuit (113). However, EC membrane proteins inhibit awkward connections between venous, arterial, and lymphatic vessels (97).

Another form of angiogenesis is intussuspective angiogenesis, where vessel walls protrude to the vessel lumen until ECs on opposite sides of the vessel come to direct cell-cell contact. After this contact, the vessel in a way pinches it self into two. Intussuspective angio-

genesis is faster and more efficient compared to sprouting angiogenesis since it does not primarily require cell migration or proliferation. The importance of this system decreases after birth but it retains a role in artery formation and vein bifurcation and trimming in adults (118).

2.2 THE ROLE OF HYPOXIA IN VASCULAR BIOLOGY

In normal conditions, the healthy individual oxygenation level is 20–70 mmHg in well-perfused tissues (119). Hypoxia exists only when oxygen levels have decreased from their initial level. Moreover, hypoxia should not be directly mixed with anoxia where there is no available oxygen or with ischemia, where the circulation to the tissues is restricted.

Hypoxia can result from: (**1**) an external hypoxic environment; (**2**) a diminished capacity of the blood to transfer oxygen, for example during carbon monoxide poisoning; (**3**) decreased cardiac output; (**4**) incapability of cells to use available oxygen for ATP production, for example during cyanide poisoning or; (**5**) in tissue level from diminished circulation, increased cell number and metabolic activity, for example during inflammation or tumor growth. For example, atherosclerotic plaque formation leads to arterial wall thickening, which can decrease the oxygen diffusion from micro vessels(120)Hypoxia is needed for normal angiogenesis, but especially during wound healing since hypoxia increases fibroblast proliferation, VEGF secretion and collagen synthesis (121).

A decrease in free oxygen concentration affects cellular respiration and cells then need to take further actions to maintain cellular homeostasis. Hypoxia is known to suppress RNA synthesis at a global level, as well as suppress several non-essential cellular functions (122). However, at the same time hypoxia activates the expression of selected genes in cells in order to improve their function under limited oxygen supply (123). In general, cell metabolism is shifted to favor anaerobic glycosylation by increasing the expression of glycolytic enzymes and glucose transporters (124) while inhibiting the protein expression involved in oxygen dependent cell respiration (125). Hypoxia is known to activate several signaling pathways but the primary mechanism for oxygen sensing at the cellular level remains unknown.

One possible mechanism of responses to hypoxia is oxygen sensing through iron-dependent prolyl hydroxylases (PHD). These hydroxylases control hypoxia inducible factor (HIF) protein expression levels. HIF proteins are transcription factors, which induce the expression of several hypoxia-response genes (126). Another, suggested mechanism for cellular oxygen sensing relates to the formation of reactive oxygen species by mitochondrial complex III since reactive oxygen species are able to stabilize HIF protein expression in a similar manner to the PHDs (127). In addition, mitochondrial cytochrome C is able to sense changes in cellular redox potential and activate several transcription factors accordingly, including the HIF family members (128). Furthermore, hypoxia is known to activate different intracellular protein kinases, such as protein kinase A and C (PKA/PKC), calcium/calmodulin-dependent protein kinase, C-Jun N-terminal kinase (JNK) Src, p38, and

p42/44 (129). In endothelial cells, HIF-1 activates the transcription of several growth factors, such as VEGF, receptor tyrosine kinases, G-protein receptors, and other signaling molecules (123).

In the vasculature, hypoxia causes vasoconstriction partly through decreasing the expression of endothelial nitric oxidase synthase (eNOS) by Rho-kinase activation (130). Hypoxia has been reported to induce ATP release from vascular endothelial cells (131), which could balance the vasoconstriction through NO release and vasodilation (132). Interestingly, a recent study shows evidence that hypoxia would actually block Cx43-mediated ATP release (25), suggesting alternative routes for ATP release in endothelial cells under hypoxic conditions. HIF activation in hypoxic tissues aims to increase oxygen availability by activating erythropoiesis and angiogenesis and at the same time by increasing vascular permeability and vasodilation (123).

Hypoxia activated HIF is closely linked to inflammation, since it can regulate innate immune responses in dendritic cells, mast cells and epithelial cells (133), neutrophils, and macrophages (134). In addition, nuclear factor κB (NFκB), which has a key role in immune response regulation, binds to HIF-1 promoter and is activated in inflammation, but also under hypoxia (135, 136). Interestingly, HIF-1 is then again able to activate NFκB transcription activity, which indicates that these two transcription factors have a co-operative role in gene expression regulation during hypoxia and inflammation (137-139).

2.3 PATHOLOGICAL VASCULAR REMODELING

Pathological vascular remodeling is often observed during or after ischemia, hypoxia, inflammation, transplant rejection, hypertension, atherosclerosis, restenosis, neoplasia, and vascular insufficiency (140, 141). It is not completely known what leads to or initiates the process of pathological vascular remodeling, but in general all modifications leading to insufficient or excess circulation can potentially be pathological. Sustained imbalance between vascular structure activators and inhibitors, cell proliferation/apoptosis, or extracellular matrix production/hydrolysis (Figure 5) can lead to pathological conditions.

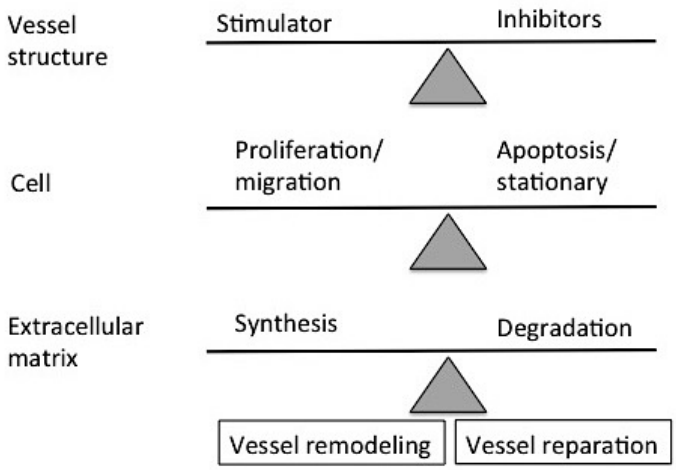

Figure 5. The balance between vessel remodeling and vessel reparation depends on the balance in: (1) vessel structure stimulators and inhibitors; (2) cell proliferation and apoptosis, and; (3) extracellular matrix synthesis and degradation. The imbalance in any of these systems can result in pathological vascular remodeling (142).

Remodeling targeting vessel structures is called angiogenic remodeling, which refers to a process where the existing vessel networks change. This includes removal of unnecessary vessels (pruning), vessel enlargement, and connective branching between vessels. Besides angiogenic remodeling, vessel thickening and loss of elasticity are forms of remodeling. All layers of vessel (tunica intima, tunica media, and tunica adventia) can thicken independently or together, which hinders blood flow though the vessel (143). This thickening is driven by excess cell proliferation, migration, and extracellular matrix production. One of the major theories about all forms of remodeling is EC dysfunction. Dysfunctional ECs are considered to switch their phenotype towards an activated and more proliferative and resistant to apoptosis compared to healthy cells (144). At this stage ECs are known to up-regulate intercellular adhesion molecule 1 (ICAM-1) expression, and the secretion of multiple pro-angionegic cytokines such as VEGF, prostaglandin E_2, interleukins (IL) 1 and 6, and ATP (140, 144). Vascular remodeling is observed during chronic hypoxia and pulmonary arterial hypertension but the concepts are basically the same in many other conditions (145).

Chronic hypoxia can cause vascular remodeling and chronically increased pulmonary artery (PA) blood pressure but the severe condition of pulmonary artery hypertension (PAH) can arise due to several other secondary reasons or with no apparent reason (idiopathic form) (146, 147). In all applicable animal models where hypoxia induces vascular remodeling there often is muscularization of previously non-muscularized vessels, medial, adventitial and intimal thickening, and loss of small blood vessels (140). All of these are also present in the classical human PAH pathobiology described in more detail in chapter 1.4 (148). Hypoxia drives the thickening of vessel intima through increased EC/SMC volume (hyper-

trophy) and increased EC/SMC number (hyperplasia)(149-151)Moreover, the SMC migration to vessel intima is known to induce intimal hyperplasia in pathological conditions such as atherosclerosis and PAH (152, 153). In addition to numerous growth factors, in hypoxic conditions ECs secrete more extracellular matrix proteins such as laminin, fibronectin, and elastin, which further affect both EC and SMC function (154, 155). The muscularization of small arteries is thought to proceed through SMC-like cells such as pericytes (156). Interestingly, advential fibroblasts appear to be the first to react to hypoxia and secrete ECM proteins, growth factors, and inflammatory cytokines, which modulate vascular structure (157, 158).

2.4 ROLE OF PURINERGIC SIGNALING IN VASCULAR HOMEOSTASIS AND REMODELING

Extracellular ATP signaling was first described in neuromuscular transmission in 1972 (159). Since then the interest towards purinergic signaling has grown considerably and purines have been demonstrated to play a significant role in the regulation of vascular tone and remodeling. It has been shown that ATP is released with noradrenaline (NA) from perivascular sympathetic nerves that mediate SMC contraction (160). However, ATP released from sensory-motor nerves can dilate some vessels(160). Besides nerve cells, ATP is also released by ECs, which leads to NO release through P2-receptor activation and subsequently to vasodilation (161). Extracellular ATP is rapidly metabolized to adenosine, which can then bind to P1 receptors in SMCs to further promote vasodilation in an NO-independent manner (162).

In HUVECs, ATP induces cell adhesion, spreading, and migration by regulating cytoskeletal rearrangement (163-165). Some of these ATP-mediated effects could be transmitted through integrins since ATP activates integrin signaling pathways, which have a central role in cell-ECM adhesion, cell migration, and angiogenesis (166, 167). In addition to HUVECs, ATP also induces cell proliferation in ECs from the aorta, coronary vessels, cornea, and pulmonary vessels (168-170). Collectively this could be interpreted to mean that ATP is a universal growth factor for ECs, somewhat similar to VEGF. Interestingly, not only ATP but also its metabolites ADP and adenosine induce EC proliferation and migration (168). Moreover, the concept that cell proliferation and apoptosis are inversely linked has gained acceptance (168, 170, 171). In smooth muscle cells, both ATP and ADP induce cell proliferation and migration but in contrast to ECs, adenosine inhibits SMC growth through A_2 receptor activation (172-174). Interestingly, EC-derived ATP can stimulate SMC migration to the vessel intima after vascular injury contributing to vascular remodeling (174).

ATP and P2 receptor signaling are well-known factors promoting vascular inflammation and leakage during hypoxia or inflammation associated conditions (175). Adversely, adenosine signaling through A_2 receptors might have a protective role in reducing vascular leakage and inflammation (176-178). Extracellular nucleotides are able to activate several intracellular signaling pathways with similar outcomes to VEGF signaling (179, 180). The heterogeneity of purine receptors on vascular endothelial cells (181) makes it challenging to

draw generalizations for all vessels; particularly pulmonary vasculature, since its endothelial cell layer is considerably different from other vessels (182).

ATP/adenosine signaling has been shown to play a role in several pathological conditions involving vascular remodeling. These include atherosclerosis, restenosis, angiogenesis, hypertension, ischemia, thrombosis, diabetes, and preeclampsia (168, 183, 184). The common denominator in all of these conditions is a shift from normal extracellular purine homeostasis, which affects vascular tone and can proceed to abnormal vascular remodeling through vascular cell proliferation and migration. It has been suggested that purinergic signaling could coordinate the complicated and dynamic processes requiring EC proliferation, differentiation, interplay with other cells, and extracellular matrix. At least some evidence of this has been obtained with $CD39^{-/-}$ mice, which are unable to form new blood vessels through angiogenesis (185).

The current knowledge of the role of purinergic signaling in vascular homeostasis is mostly limited to systemic vasculature studies. Some publications on pulmonary vasculature in diseased states involving purinergic signaling exist as well. In pulmonary hypertension (PH), ATP-$MgCl_2$ has been used as a vasodilator to treat patients, however due to its rapid metabolic rate, it is not optimal for long-term treatment(186)In addition to ATP, adenosine has also been used to treat increased vascular resistance and pressure in PAH and in chronic obstructive pulmonary disease (COPD) patients (187, 188). Moreover, adenosine receptor agonists have been used successfully as a treatment in the rat model of PAH (189).

At the systemic level, it has been shown previously that pulmonary adenosine levels are decreased in PAH and COPD patients (190). At the cellular level, the mitogenic effects of ATP to pulmonary SMCs are considered to have a substantial role in the severe forms of vascular pathology (191). After an acute injury to the vasculature, accumulation of ATP could be beneficial to quickly improve vascular barrier function (192) and it is probable that the long-term accumulation causes the observed changes in vascular structure. Likewise, long term accumulated adenosine concentration can be harmful for ECs (193). Accumulation of ADP can also be harmful for the vasculature since it induces cell proliferation but it is also a potent activator of platelets (194). This is important since thrombosis is a severe form of vascular remodeling and it is seen in patients with severe PAH (195).

2.5 PULMONARY ARTERIAL HYPERTENSION

The classifications describing the different forms of PH were first created at a World Health Organization (WHO) symposium in 1973 and the most recent modifications to these classifications were made during the 5th World Symposium in 2013 (196, 197). PH, which should not be mixed with systemic hypertension, is an umbrella term for numerous conditions where blood pressure in the pulmonary circulation is increased. Different forms of pulmonary hypertension are divided to: (**1**) pulmonary arterial hypertension (PAH); (**2**) PH due to left heart disease; (**3**) PH due to chronic lung disease and/or hypoxia; (**4**) chronic thromboembolic PH, and; (**5**) PH due to unclear multifactorial mechanisms.

PAH is defined by increased pulmonary artery pressure (>25 mmHg), normal pulmonary artery wedge pressure (<15 mmHg), and increased vascular resistance (>3 Wood units)(198, 199)For clinical purposes PAH is further subdivided to four groups: (**1**) Idiopathic PAH, which can occur with out any identifiable cause for the disease; (**2**) Heritable PAH that is a hereditary form of PAH; (**3**) Drug and toxin induced PAH; (**4**) PAH associated with connective tissue disease, HIV infection, portal hypertension, congenital heart diseases, or schistosomiasis (197).

The idiopathic form of the disease is rare in the general population; only 2-3 patients per million per year are diagnosed (200). The global population was approximately 7.2 billion in 2013, which would account for roughly 18 000 newly diagnosed patients suffering from iPAH in 2013. Patients diagnosed for iPAH are most frequently 30–40 year old females with symptoms such as dyspnea on exertion (difficulty in breathing), fatigue, syncope (fainting), leg edema (swelling), and palpitations (abnormality of heartbeat). The average life expectancy after diagnosis is quite short, three years without medication and around ten years with medication. This is because the symptoms come only at very late stage of the disease and because iPAH is hard to correctly diagnose (196).

There are no efficient treatments for iPAH except full heart and lung transplantation in selected cases (196). Currently there are three classes of medical therapies to treat PAH: prostacyclin analogues, endothelin receptor antagonists, and phosphodiesterase 5 (PDE5) inhibitors that all function through inducing vasodilation (201). One of the interesting novel therapeutic candidates to treat PAH is apelin. Apelins are ubiquitously expressed small peptides, which are known to bind specific APJ G-protein linked receptors (202). Moreover, it is well established that the apelin-signaling pathway is disrupted in PAH. Human patients and animal models of PAH have been linked to a dysfunctional SMC and EC phenotype (202). Even though the mechanism is not fully understood, apelin administration has been successfully used in animal models of PAH, but its clinical use requires further investigation (202).

The initial cause of idiopathic PAH is unknown. The vascular changes (Figure 6) observed in pathological studies form PAH patients include vasoconstriction, medial and intimal thickening due to induced cell proliferation, muscularisation of arterioles, dramatic decrease in the number of microvessels, and complex and obscure structures called plexogenic lesions that radically inhibit blood flow (203). Pulmonary vasoconstriction is believed to be the initial trigger for more severe vascular remodeling (204). Remodeling and proliferation of all vascular layers occurs during PAH pathogenesis (205). Even though the pathogenesis of PAH is a multifactorial condition, ECs have a central role in the onset of vascular remodeling (206).

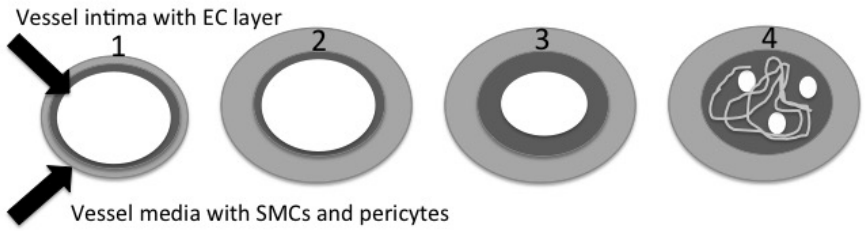

Figure 6. Remodeling of arteries during PAH pathogenesis occurs in many forms. (1) Increased vascular resistance causes vasoconstriction. (2) Abnormal muscularization of small arteries, medial hypertrophy and hyperplasia. (3) Vessel occlusion caused by neointimal formation. (4) Formation of aberrant endothelial cell channels to the otherwise closed vessel that are called plexiform lesions.

EC dysfunction and/or malfunction of potassium channels are considered to be the reason for the initial vasoconstriction leading to PAH (204, 205). In familial and idiopathic forms of PAH, some genetic predispositions have been identified. One of the major hallmarks is bone morphogenetic protein receptor 2 (BMPR2) (196). This serine/threonine receptor kinase binds to bone morphogenic proteins, which were first identified by their ability to induce bone and cartilage growth (207). Mutations in this receptor have been identified in 60% of familial cases and in 10–30% of sporadic cases of PAH (208-210). Furthermore, patients without a BMPR2 mutation also have markedly decreased BMPR2 protein expression level (211). One PAH treatment option would be be to stimulate BMPR2 expression, and for this tacrolimus seems to be a promising candidate (211). Conversely, BMPs belong to the vast TGF-β superfamily that has several potential roles in vascular remodeling related to PAH. These roles include: collagen and matrix deposition, chemotaxis, inhibition or stimulation of proliferation, regulation of other vasoactive substances, cell differentiation, modulation of immune cell function, apoptosis inhibition, and activation of proteolytic enzymes (204).

2.6 ENDOTHELIAL CELL DYSFUNCTION AND GENOMIC INSTABILITY

According to the current understanding, EC layer injury, hypoxia or shear stress could activate the normally quiescent state ECs. This activation could induce the up-regulation of several key vasoactive factors such as: ATP, prostacyclin, endothelin-1, thromboxane, VEGF, polyamines, and xanthine oxidoreductase (206). In addition to growth factors, ECM elements secreted from the stressed ECs could further activate the nearby SMCs (212). Prolonged cell activation or severe injury may lead to EC dysfunction. This enhances thrombosis through the up-regulation of selectins, thrombomodulin, and von Willebrand factor (213). Moreover, the normal barrier function of dysfunctional ECs is greatly compromised, which facilitates the diffusion of growth factors to the sub-endothelium propagated by the

abnormal eNOS function leading to increased or decreased NO production, which regulates vasodilation (206). While some cells die upon severe damage, many of the surviving cells become dysfunctional. Genetic predispositions could offer a partial explanation to this since mutation in genes, such as BMPR2 has been for example shown to render ECs more prone to apoptosis (214). In addition, the gene coding for transforming growth factor-beta (TGF-β) receptor has also been linked to endothelial dysfunction (215).

During PAH pathogenesis the initial EC apoptosis is followed by the rise of an apoptosis-resistant and hyperproliferative cell population (216, 217). This cell population is characterized to have major chromosomal instability and decreased ability for DNA repair (218, 219). Moreover, a recent metaanalysis revealed that many of the key DNA-repair associated genes are also dysregulated in BMPR2 deficient ECs (219). The functional role of the DNA repair associated genes was also confirmed *in vitro* by showing that BMPR2 deficient ECs had increased susceptibility to irradiation and oxidative-stress induced DNA damage (219). This phenotypic change was consistent with pulmonary ECs from PAH patients. BMPR2 expression is rapidly downregulated upon DNA damage accumulation in a study by Li et al.. This led the authors to suggest a compelling theory that the prosurvival BMPR2 is silenced after cell damage to inhibit cell survival and to promote apoptosis. The mechanism by which thw hyperproliferative EC population gets past this regulation is not fully understood. Interestingly, the loss of BMPR2 expression correlates with more aggressive carcinomas implying that BMPR2 also has a regulatory function in some cancer cells (220, 221).

Chromosomal abnormalities in PAH patients have also been demonstrated with histological samples (222, 223). It has been recently shown that the observed chromosomal rearrangements, DNA damage level, and mutagen sensitivity do not correlate with any known mutation, associated with hereditary PAH, but these events seem consistent in all PAH cases (224). During PAH pathogenesis, the increased reactive oxygen species (ROS) in pulmonary ECs that appears to correlate with elevated DNA damage level, as opposed to genetic influence (219, 224). In the case of PAH, chromosomal abnormalities may even enhance cell proliferation though DNA damage, whereas the usual impact is to stall cell proliferation and potentially trigger apoptosis. In deed, a large interstitial deletion of chromosome 13 has been identified to play a role in the hyperproliferative phenotype of dysfunctional ECs (222).

Even though chromosomal abnormalities are highlighted here, PAH-patient pulmonary ECs are still largely normal when compared to cancer cells (224). DNA integrity is highly important since the hereditary information is needed for protein production. The hereditary information is stored in genes, which determine the characteristics of every cell, individual, and species. Besides endogenous DNA damage several chemicals and irradiation are known to be harmful for DNA integrity. One of the most used animal models of PAH is the monocrotaline (MCT) rat model where single injection of MCT triggers the condition. It is also known that MCT is a highly genotoxic substance, which induces DNA crosslinks (225). Moreover, several genes involved in DNA repair were downregulated in a global gene expression profiling of pulmonary ECs isolated from MCT treated animals (219). To-

gether this suggests that genomic instability is also an important feature in MCT induced PAH.

Several cancer therapies are designed to disrupt the DNA integrity of cancer cells, which have very dysfunctional DNA repair mechanisms when compared to healthy cells. However, non-malignant cells are also exposed to these treatments to some extent. This could explain that while the survival rate of cancer patients has greatly improved over the last few decades, the incidence of cardiovascular diseases in these survivors has also increased (226, 227). The reasoning for this could be a DNA-damage-induced dysfunctional EC phenotype that can lead to vascular remodeling as observed in PAH. Moreover, there is a growing body of direct evidence that chemotherapy and irradiation may trigger PAH (228, 229).

AIMS OF THE STUDY

Pathological vascular remodeling and angiogenesis play important roles in a variety of conditions, such as systemic and pulmonary hypertension, chronic periodontal disease, chronic kidney disease, diabetes, atherosclerosis, postangioplasty restenosis, vascular insuffiency, benign neoplasia, and cancer. All of these conditions are associated with ischemia, hypoxia and inflammation. This dissertation project focuses on the involvement of purinergic signaling, especially CD39, governed ATP hydrolysis and ATP mediated signaling, in EC dysfunction and the resulting vascular remodeling. Major aims and questions to be addressed in this study can be summarized as follows:

1. We aimed to develope a novel sensing method for simultaneous detection of nanomolar concentrations of ATP and all its dephosphorylated metabolites in the same sample. (IV)

2. How are the extracellular purinergic signaling and metabolic pathways shifted during exposure of vascular ECs to hypoxia? (I)

3. Is the vascular remodeling observed in PAH patients connected to purinergic signaling and could it be used as a therapeutic target? (II)

4. Could ATP-mediated signaling be linked to DNA damage repair and could it be used to pre-treat cells prior to irradiation or chemically-induced DNA damage? This information could be useful in cancer therapy. (III)

MATERIALS AND METHODS

4.1 ETHICAL CONSIDERATIONS (I–IV)

All experimentation with male Holstein calves in the first study was done according to institutional guidelines at the Department of Physiology, School of Veterinary Medicine, Colorado State University. In addition, the Finnish National Animal Experiment Board approved all animal experiments in studies II and III. The use of human derived samples used in study II was accepted by the appropriate Institutional Review Board on human subjects according to the declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects. The primary endothelial cells and lung paraffin sections from iPAH patients were from the tissue center of the Cardiovascular Medical Education and Research Fund – Pulmonary Hypertension Breakthrough Initiative (CMREF–PHBI).

4.2 CELL ISOLATION AND CULTURE (I, II, III, IV)

Table 1. All used cell lines at glance.

Cell type	Source	Product #	Abbreviation	Paper
Calf vasa vasorum EC	Isolated	-	VVEC	I
Human umbilical vein EC	Isolated	-	HUVEC	I, IV
Human dermal microvascular EC	PromoCell	C-12210	HDMEC	IV
HUVEC-carcinoma fusion cell line	ATCC	CRL-2922	EAhy926	IV
Human leukemic T-cell lymphoblast line	ATCC	TIB-152	Jurkat	IV
Human prostate carcinoma cell line	ATCC	CRL.1435	PC3	IV
Human breast cancer cell line	ATCC	HTB-26	MDA-MB-231	IV
Mouse melanoma cell line	ATCC	CRL-6475	B16-F10	IV
Lung microvascular EC from iPAH patient	Isolated	-	iPAH EC	II
Human microvascular EC (lung)	Lonza	CC-2527	HMVEC	II
Human pulmonary arterial SMC	PromoCell	C-12522	SMC	II
Human pulmonary microvascular EC	PromoCell	C-12281	HPMEC	III
Human chronic myelogenous leukemia cells	Sigma	89121407	K562	III
Human diffuse large B-cell lymphoma cells	ATCC	CRL-2957	SUDHL-4	III

All cell types used in studies I–IV are summarized in table 1. Vasa vasorum endothelial cells (VVEC) from normoxic (control) and chronically hypoxic male Holstein calves were isolated as previously described (230). The animals in hypoxic group were kept in hypobaric hypoxia (P_B = 430 mmHg) for two weeks prior the animals were euthanatized. Endothelial cells were isolated from the adventitial compartments and purified from adventitial fibroblasts with cloning rings and trypsinization techniques. The VVECs were provided by Dr. Evgenia Gerasimovskaya (University of Colorado, Denver) on collaborative basis and cultured in high glucose Dulbecco´s modified Eagle-medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM L-glutamin, all from Lonza (Walkerville, MD, USA). During the cell stock culturing 30 µg/ml Cell Growth Supplement (ECGS, Millipore, USA) was used to enhance cell proliferation.

Human umbilical endothelial cells (HUVECs) were isolated from fresh human umbilical cords according to previously published method using Collagenase I to detach the EC lining from the umbilical cord (91). Human primary microvascular ECs from the lung (HMVEC, Lonza) and human pulmonary microvascular ECs (HPMEC, PromoCell) both from three different donors and pulmonary microvascular ECs derived from two iPAH patients were commercial cell lines. All mentioned endothelial cell were grown in EBM-2 basal medium supplemented with EGM-2 bullet kit (Lonza). Cell culture dishes were coated with 0.2% gelatin. Cells were sub cultured at a 1:4 ratio after they reached above 80% confluence and used in experiments at passages 3-10.

Commercial Human dermal micro vascular endothelial cells (PromoCell GmbH, Heidelberg, Germany) were cultured in media provided with the cells (Growth medium MV with Supplement Mix). Fusion cell line EAhy926 derived from HUVEC and human lung carcinoma cell line A549 and human leukemic T-cell lymphoblast line Jurkat (clone E6-1) from ATCC (Manassas, USA) were cultured in RPMI-1640 supplemented with 10% FBS, 4mM L-glutamine, 1mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Breast cancer cell line MDA-MB-231 and mouse B16-F10 melanoma cells from ATCC were cultured in Dulbecco´s Modified Eagles medium supplemented with 10% FBS, 4mM L-glutamine, 1mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Prostate carcinoma cell line PC3 cells were also from ATCC and cultured in Ham´s F-12K medium Gibco (Life Technologies) supplemented with 10% FBS. Human pulmonary artery smooth muscle cells (PromoCell) were cultured with SmGM-2 BulletKit (Lonza). Human chronic myelogenous leukemia and human diffuse large B-cell lymphoma cells (ATCC) were both cultured in RPMI-1640 media (Sigma) supplemented with 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin.

Rat pulmonary microvascular ECs were isolated essentially as described earlier (219, 231). The whole lung was dissected from the animals and the tissue was minced to small pieces. Cells were detached from the tissue with Collagenase digestion (Collagenase IV (Sigma) 1.0 mg/mL and DNase I (Sigma) 7 µg/mL). Tissue homogenate was incubated for 45 minutes at 37°C with shaking. After this the homogenate was filtered through 70 µm pore size nylon cell strainers and the flow through was centrifuged. The cell pellet was incubated

in Buffer 1 (PBS, 1% bovine serum albumin (BSA) and 2 mM EDTA) with goat anti-mouse magnetic beads (Dynabeads, Invitrogen, Carlsbad, CA, USA) coated with mouse anti-rat CD31 (BD Pharmingen, San Jose, CA, USA) to pull down ECs for experimentation. Isolated cells were washed and pulled down with a magnet (Invitrogen, DynaMag™) and finally suspended to 200 µL RPMI 1640 and used for CD39 ecto-ATPase activity measurement.

The human primary pulmonary microvascular ECs from two iPAH patients were isolated essentially similarly as cells from the rats. Dissected pieces of lung tissue were minced to small pieces and digested with collagenase IA (Sigma 1 mg/ml). Mixture was incubated for 45 minutes at 37°C, after which the cell suspension was filtered and centrifuged. The pellet was incubated with human CD31 antibody coated magnetic beads (Dynabeads, Invitrogen). Isolated cells were pulled down with a magnet suspended to EGM-2 culture media(231)All cells were cultured in a standard cell incubator at 37 °C in a humified athmosphere and 5% CO_2.

4.3 PAH AND DNA DAMAGE RAT MODELS (II, III)

(II) Animals used in experiment (Spraque Dawley, 5 weeks old) were given a subcutaneous injection of monocrotaline (MCT, Sigma, 60 mg/kg) or physiological salt solution (N=7). Five days after injection the animals were euthanatized with CO_2 followed by exsanguination. The vascular ECs were isolated from the dissected lungs. In separate experiment, rats (N=5) were given subcutaneous injection of MCT or PBS (at day 2) with the difference to the first experimental setting that animals were at the same time treated with intraperitoneal injections of PBS or apelin-13 peptide (1 mg/kg, American peptide company, Sunnyvale, CA, USA) starting from day 1. Five days after the MCT injection animals were euthanatized and the ECs isolated from the lungs as previously.

(III) The used animals (N=3) were given sodium polyoxotungstate POM-1 (Tocris) (10 mg/kg, i.p.) or PBS in three consecutive days followed by MCT injections 60 mg/kg at day three. The next day animals were euthanatized and samples for Western immunoblotting and immunohistochemistry were collected. In the second animal experiment the rats (N=3) were treated with POM-1 or PBS as before but instead of MCT the rats were given doxorubicin hydrochloride (Tocris 6 mg/kg, i.p.). The heart samples were collected 8h after doxorubicin injection and processed for Western immunoblotting and immunohistochemistry samples.

4.4 THIN LAYER CHROMATOGRAPHIC ANALYSIS OF PURINE CONVERTING ECTO-ENZYMATIC ACTIVITIES (I, II)

Purine converting ecto-enzymes were assayed at +37°C and experimental conditions were optimized for each sample set separately. We used tritium labeled substrates (ATP, ADP and AMP) and measured the amount of hydrolyzed products with liquid scintillation β-

counter. 4 mM β-glycerophosphate (Sigma) was added to all reaction mixtures in order to prevent undesirable hydrolysis of ^{3}H -nucleotides by non-specific phosphatases. Substrate concentrations and incubation times were selected so that the amount of formed products would not exceed 15% from the initial amount of substrate. Reactions were stopped by applying 8 µl aliquots onto thin-layer chromatography (TLC) plates (Macherey-Nagel Alugram® Sil G/UV_{254}, Germany). The TLC method was used to separate ^{3}H-labelled substrates and their metabolites. On TLC plates ATP and its metabolites move at different rates when the running phase is optimized (Figure 7).

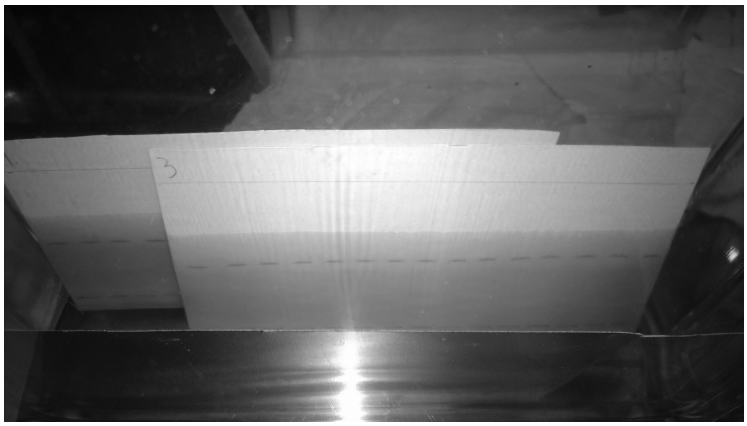

Figure 7. Samples applied to TLC-plates running in a glass tank with solvent based running phase on the bottom of the tank.

Standards (1 mM ATP, ADP, AMP, adenosine, inosine and hypoxanthine) were applied to TLC plates before samples were added. Standards were used to make the bands visible under UV-light irrespective of the amount of formed purine metabolites. As running phase with TLC-plates a specific solvent mixture: 1-butanol, iso-amyl alcohol, diethylene glycol monoethylether, ammonia solution and milli-Q-aqua (9:6:18:9:15) was used (232). Bands containing the substrate and formed metabolites were visualized under UV-light and collected to scintillation vials with 0.1M or 0.2M HCL. Scintillation cocktail was then added to samples (Optiphase Hisafe 2 or 3, PerkinElmer). Samples were quantified with either Wallac-1409 liquid scintillation β-counter or MicroBeta2 Plate counter (PerkinElmer). Specific enzyme activities were calculated from raw data gathered from β-counter (Formula 1).

Formula 1.

$$A = \frac{(Smpl - Bl)\times 60\times [Subst.]\times AV\times 1000}{Total\times t\times s}$$

Smpl = ^{3}H - Counts from the sample (dpm)
Bl = ^{3}H - Backround (dpm)
[Subst] = Substrate concentration (μM)
Total = Total ^{3}H - Counts added to the well (dpm)
t = Incubation time (min)
s = Mixture aliquot added to TLC-plate (μl)
AV = Total reaction volume (ml)

Specific conditions in each assay:

(I) eNTPDase1/CD39, the VVECs and HUVECs were incubated for 20−25 min with 500 μM ATP (Sigma, Germany) with tracer [2,8-^{3}H]ATP (spec. act. 19.0 Ci/mmol, PerkinElmer, USA) or ADP (Sigma) with tracer [2,8-^{3}H]ADP (spec. act. 35.1 Ci/mmol, PerkinElmer). 5′-NT, cells were incubated for 45 min with 300 μM AMP (Sigma) with tracer [2-^{3}H]AMP (spec. act. 19.7 Ci/mmol, Quotient Bioresearch, GE Healthcare, UK). AK and NDPK, cells were incubated with 500 μM [^{3}H]AMP (for 45 min) or [^{3}H]ADP (for 10 min) as respective phosphorus acceptors and 700 μM of γ-phosphate-donating ATP.

(II) eNTPDase1/CD39, human ECs and ECs isolated from the rat lungs were incubated with 350 μM ATP with tracer [2,8-^{3}H]ATP (PerkinElmer) for 25 min. Part of the in vitro assays were made with 1 μM apelin-13 peptide pre treatment (30 min–o/n).

(IV) eNTPDase1/CD39, cells were incubated on 24-well plates with 10 μM ATP (Sigma) with tracer [2,8-^{3}H]ATP (PerkinElmer) or (ADA) with 10 μM adenosine (Sigma) with tracer [2-^{3}H]adenosine (Amersham Biosciences). ^{3}H-labelled nucleotides and nucleosides were separated and quantified by TLC as described above.

For better visualization of the quantitative data on enzymatic activities, autoradiographic analysis for of major purine converting pathways was used. In addition, with this method also the accumulated inorganic Pi and PPi metabolic products from the nucteotide hydrolysis can be detected. This information can be used to determine the key nucleotide hydrolysing enzymes.

(I) TLC-plates were exposed to Kodak BioMax maximum sensitivity films for at least two weeks at -70°C, which were then developed by autoradiography. To confirm which enzymes are responsible for the particular catalytic reaction specific enzyme inhibitors were

used. Specifically, for 5´NT activity inhibition 10 μM adenosine α,β-methylene diphosphate (AMPCP, Sigma), for AK inhibition 10 μM Ap_5A (Sigma) and for ADPase inhibition 10 μM sodium polyoxotungstate (POM-1, Tocris Bioscience, UK). The nucleotide metabolism of VVECs was evaluated with autoradiography also by incubating cells with 20 μM [γ-^{32}P]ATP (PerkinElmer) for 45 min. Metabolites were subsequently separated with CEL-300 PEI TLC-plates (Macherey-Nagel) with 0.75 KH_2PO_4 (pH 3.5) as running phase.

(IV) Similar autoradiographic analysis was done as in project I. by incubating PC3 and MDA-MB-231 cells with 20 μM [γ-^{32}P]ATP for 20 min.

4.5 QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (I,II)

Specific primers used in each experiment are presented in table 2 and the expression of target genes was always normalized to that of β-actin (housekeeping gene). Cellular RNA from the cells of interest was isolated with RNease spin columns (Qiagen, Valencia, CA, USA)(I) or with Nucleo Spin RNA II-kit (macherey-Nagel)(II). From the isolated RNA cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA)(I) or with Superscript VILO (Invitrogen)(II). RT-PCR reactions were performed with iTaq FAST SYBR Green Supermix with ROX (Bio-Rad) (I) or with qRT-PCR SYBR green MasterMix (eurogentec, Mesa Green qPCR Mastermix Plus for SYBR assay) using ABI 7500 Fast Real-time PCR System (Applied Biosystems, Inc., Foster City, CA, USA).

Table 2. List of all primers used thise study

Gene	Forward	Reverse	Organ-ism	Pa per
entpd1	AATAAAGATGAGCGTCTTAAACGA	CCACGGATTTCAATGTCAACGAG	calf	I
CD73	TCTGAGCGCAAACATTAAAGCC	CAATCCCCACAACTTCATCACC	calf	I
HIF-1α	CTTCGGTATTTAAACCATTGCAT	GGACAAACTCCCTAGCCCAA	calf	I
β-actin	CGGTCAGGTCATCACTATCG	TTCCATACCCAGGAAGGAAG	rat	II
entpd1	GTCTCTCCTTCTGCAAGGCT	TTGCTGTCTTTGATCTTGCC	rat	II
p2ry11	GACTTCCTGTGGCCCATACT	CAGCTGGACAGAGAAGACCA	human	II
entpd1	GAGGAGCCTCAGCAACTACC	TGAATTTGCCCAGCAGATAG	human	II
β-actin	CACTCTTCCAGCCTTCCTTC	GGATGTCCACGTCACACTTC	human	II
aplnr	CCCTAAACCACAAACCTCTGA	GCTACTCCTTGTCCTATGCAC	human	II

4.6 WESTERN BLOTTING ANALYSIS (I,II,III)

All used antibodies are specified in table 3. In project I, proteins from whole cell lysates were separated on 4-12% Bis-Tris polyacrylamide gels (Invitrogen Inc, Carlsbad, CA, USA). Proteins were transferred to PVDF membranes (I) or nitrocellulose membranes (III, IV) from the gels and membranes were blocked with 5% non-fat milk or 3% BSA before

applying primary and secondary antibodies. Obtained bands were visualized with ECL kit (Renaissance, NEN Life Science Product, Boston, MA, USA or Enhanced ECL, PerkinElmer) and analyzed with ImageJ software.

Table 3. Antibodies used in immunoassays

Antibody	Host	Dilution	Type	Aplication	Source		Paper
CD39	goat	1:500	polyclonal	WB	Santa-Cruz	H-85	I
GAPDH	rabbit	1:1,000	monoclonal	WB	CST	14C10	I
P2Y11	rabbit	1:150	polyclonal	IHC	Abcam	ab140878	II
P2Y11	rabbit	1:250	polyclonal	WB	Pierce	PA5-28772	II
CD39	mouse	1:500	monoclonal	WB	Abcam	ab30422	II
β-actin	goat	1:2,000	polyclonal	WB	Abcam	ab8229	I,II,III
ERK1/2	rabbit	1:200	polyclonal	WB	Santa-Cruz	sc-94	II
pERK1/2	mouse	1:200	monoclonal	WB	Santa-Cruz	sc-7383	II
pH2A.X		1:5,000		WB			
pH2A.X		1:100		ICC			
pH2A.X	mouse	1:1,000	monoclonal	IHC	Millipore	MABE205	III
p53	rabbit	1:1,000	polyclonal	WB	CST	9284	III
CD39	goat	1:200	polyclonal	IHC	Santa-Cruz	H-85	II
CD31	mouse	1:50	monoclonal	IHC	CST	89C2	II
CD34	mouse	1:50	monoclonal	IHC	CST	ICO115	II
CD39	mouse	1:1,000	monoclonal	iF	Ectonucleo-tidases-Ab	hN1-9$_{L(I_4,I_5)}$	II

4.7 BIOLUMINESCENT QUANTIFICATION OF EXTRACELLULAR ATP, ADP AND PPi (I,II,IV)

(I) The extracellular levels of ATP, ADP and PPi were also determined with a luminescence-based assay. In project I, VVECs cultured in 24-well plates were washed before the cell media was changed to Krebs-Ringer phosphate glucose (KRPG; 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose; pH 7.35). Sample aliquots (100 µl) were collected from the wells after 1 and 2.5 h of incubation at conventional cell incubator. The ATP and ADP concentrations were quantified from these samples using ATPlite kit (PerkinElmer). Samples from the aliquots were transferred to 96-well plates with H_2O (mixture A) or with 200 µM UTP and 5 U/ml NDP kinase (Sigma) (mixture B) and proceeded with the protocol according to manufacturer's instructions. Luminescence from the samples was read with Tecan Infinite-M200 (Salzburg, Austria). ATP concentration from A samples and ADP concentration by subtracting the signal A from B samples (B-A=[ADP]) were quantified from calibration curves.

(II) The rate of 2 µM extracellular ATP clearance with iPAH and control ECs were analyzed as in project I with phosphate free basal salt solution (BSS: 130 mM NaCl, 5 mM

KCl, 1.5 mM $CaCl_2$, 1mM $MgSO_4$, 25 mM HEPES, 5 mM glucose and 0.1% bovine serum albumin (BSA), pH 7.4). Parts of these assays were made with1 μM apelin pretreatment.

(IV) In project IV, ATP and ADP concentrations were measured as in project I with KRPG buffer and with chemical treatments. The following drugs were used: pro-inflammatory cytokine human recombinant tumor necrosis factor-α (TNF- α, 200 U/ml), interleukin 1β (500 U/ml) (both from R&D Systems), endotoxin lipopolysaccharide from Escherichia coli 0111:B4 (LPS, 10 ng/ml), non-hydrolysable cAMP analogue dibutyryl cAMP (20 μg/ml), and inhibitor of nucleoside transport S-(4-nitrobenzyl)-6-thiosine (NBT, 1 μM) (all from Sigma). In project IV measurements were done with BSS with or without 2 μM ATP or adenosine. With similar luminescence based assay the concentration of formed extracellular PPi was also quantified. The protocol was adapted from previously published enzyme-linked bioluminescence assay (233) producing ATP, which could be measured with the ATPlite kit with 5x concentrated ATP-monitoring reagent. By subtracting the signal from ATP samples from PPi samples the extracellular PPi concentration could be quantified.

4.8 TRANSENDOTHELIAL PERMEABILITY ASSAY (I)

HTS FluoroBlok™ 96-Multiwell Insert system with 8 μm pore size (BD Falcon) and FITC-conjugated dextran 70 kDa (Molecular Probes®, Invitrogen) was used to analyse the permeability of VVEC monolayers. Wells were coated with fibronectin before cells were seeded to the inserts and cultured in complete media until near 100 confluency was formed. The integrity of the monocell layer was tested with human red blood cells. Prior to experiment, cells were washed and moved to serum free DMEM after which cells were pre-treated 30 min with 10 μM ATP, ADP, adenosine, 5´-N-ethyl-carbox-amide-adenosine (NECA), or 5´-(N-cycloprolyl)-carbox-amido-adenosine (CPCA) (all from Sigma). After the pre-incubation the experiment was initiated by applying 35 μm FITC-dextran onto the cells and its flux from upper well to lower well through the EC monolayer was monitored periodically by measuring the fluorescence with Tecan Infinite-M200.

4.9 FLUORESCENT QUANTIFICATION OF EXTRACELLULAR AMP, ADENOSINE, INOSINE, AND HYPOXANTHINE (I, IV)

For this experiment a novel sensing technique, which uses enzyme cascade to sequentially convert AMP to H_2O_2, was developed. The formed H_2O_2 can be quantified with a sensitive and long lasting probe 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), which react with H_2O_2 resulting to formation of fluorescent product resorufin. The fluorescence based assays were made from the same sample aliquots taken for biolumiscent detection of ATP (KRPG and BSS). Samples were transferred to 96-well plates with different combinations of following enzymes: 0.15 U/ml 5'-nucleotidase from Crotalus adamanteus venom, 0.3 U/ml adenosine deaminase (ADA, type IX from calf spleen), 0.25 U/ml bacterial purine nucleoside phosphorylase (PNP) and 0.15 U/ml microbial xanthin oxidase (XO); all enzymes were from Sigma. Samples were incubated with the enzymes for 20 min at RT prior

the Amplex Red (60 µM, Invitrogen, Molecular probes) and HRP (1 U/ml, Sigma) were added. Schematic presentation of the used enzyme cascade is presented in figure 8. The mix A contained all the enzymes for the entire cascade from AMP to H_2O_2, the mix B does not have the 5'NT, mix C is lacking both 5'NT and ADA, and so on up to mixture E to which no enzymes, besides the Amplex Red and HRP, were added. With calibration curves the AMP concentration was obtained with a simple equation (Signal A–Signal B = Signal from AMP). Adenosine concentration was similarly calculated (B–C) and so on for each product.

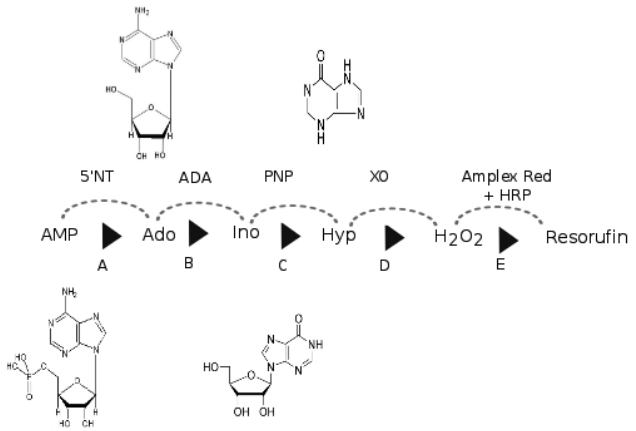

Figure 8. Enzyme-coupled purine sensing assay. With the use of different enzyme mixtures the concentration of wide range of purines could be quantified from the same samples simultaneously. The enzyme mixtures were labeled from A-E and each one had one less enzyme than the previous one creating a multi step cascade. By subtracting the following signal from the proceeding one we were able quantify products from AMP to hypoxanthine.

4.10 PROLIFERATION ASSAY (I, II)

(**I**) Seeded ECs were serum starved in DMEM growth media for 72 h prior stimulation with ATP, ADP or adenosine (1 nM – 1mM). Cells were then cultured another 24 h with 0.125 µCi of [methyl-^{3}H]-thymidine. Incubation was stopped with 0.2 M perchloric acid and lysates were mixed with liquid scintillation cocktail (Ecoscint H, National Diagnostics, Atlanta, GA, USA) to measure the incorporated ^{3}H–labeled thymidine with scintillation β-counter (Beckman LS 6500) (230).

(**II**) In the cell counting method Non-Target, CD39, and P2Y11 siRNA teated cells were seeded to 24-well plate in complete medium and let to attach. The number of attached cells in wells was counted to confirm equal seeding. The next day wells were washed with PBS and the cell culture media was changed to non-supplemented RPMI-1640 media and kept in

cell incubator for 6h. After the incubation period the wells were washed with PBS and the cell number (cells/ml) was analysed under a microscope.

4.11 cAMP ASSAY (I)

Intracellular cAMP concentrations were assayed with LANCE® cAMP Detection Kit (PerkinElmer) according to manufactures instructions. Detached VVECs were activated with G-protein coupled receptor agonists: forskolin, NECA, CPCA and adenosine (0.1 μM–100 μM) for 45 min. FRET emission of the formed cAMP complex was read at 665 nm and quench correction was made at 615 nm (Tecan Infinite M200).

4.12 EX VIVO ANALYSIS OF PURINE METABOLISM IN HUMAN UMBILICAL CORDS (I)

Freshly cut human umbilical cords were sliced to length of 8 cm. Cord slices, were then washed with Hank's salt solution and BSS. After washing we injected 2 ml of BSS, with or without 2 μM ATP or adenosine, to the cords and clamped the both ends. The clamped cords were incubated in a water bath (20 min, +37°C). After incubation the previously injected BSS buffer was carefully collected and heat-inactivated. We assayed the purine nucleoside, nucleotide and PPi concentrations as specified earlier in chapter 4.9.

4.13 RNAi (II, III)

CD39 and *P2Y11* genes were downregulated with RNAi technique. Controls were treated with non-target siRNAs (On-Target plus smart pool system-siRNAs, Dharmacon, Fischer Scientific, Vantaa, Finland), cd39 cat# L015973-00-0005, p2y11 cat# L-005691-00-0005). Transfections were made with Opti-MEM (Gibco) and Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) as described previously (234).

4.14 CELL SURVIVAL AND VIABILITY (II, III)

(**II**) Cell survival under several conditions was analyzed by measuring caspase 3/7 activities in cells. Caspase assays (Caspase 3/7 assay, Promega Nacka, Sweden) were done in 96-well plates according to the manufactures instructions. Cells were seeded in complete meidia and let to attach over night after which the media was changed to serum free EBM-2 media with treatments 2 μM ATP or 50 μM $P2Y_{11}$ agonist β-Nicotinamide adenine dinucleotide (β-NAD, Sigma). The cell viability under different conditions was assayed with a XTT assay (Cayman chemical, Tallin, Estonia) according to the manufactures instructions. For the XTT assay cells were seede in complete media to 96-well plates and let to attach over night after which the media was changed to EBM-2 supplemented with 2% FCS with or without treatments (ATP, β-NAD). After the media change cells were incubated for another 24h before the absorbance was read.

(**III**) Cell survival of K562 and SUDHL-4 cancer cells was assayed with the same caspase 3/7 assay as before. Doxorubicin hydrochloride 1 μM (DOX, Tocris) was used up to 24h timepoint to induce caspase 3/7 activation with or without 100 μM sodium polyoxotungstate 1 (POM-1, Tocris) overnight pretreatment.

4.15 SMC MIGRATION THROUGH POROUS MEMBRANE (II)

Assays were done using Transwell® Permeable Supports with 5 μm pore size (Corning, Amsterdam, The Netherlands) according to the manufacturer´s instructions. ECs with 2 μM ATP or 1 μM apelin in the bottom wells were used to induce or inhibit the SMC migration through the membrane during over night incubation. The next day the cells that had transferred through the membrane were fixed with formalin, stained with hematoxyllin and eosin, and the number of cells was counted under a microscope.

4.16 IN VITRO INDUCED DNA DAMAGE (III)

Cellular DNA damage with ECs and with used cancer cell lines was induced to cells with γ-irradiation using 4 Gy or 5 Gy, the median irradiation dose for ECs based on the literature(235)Furthermore, DNA damage was induced with chemotoxicants Methyl methanesulfonate (MMS, 500 μM) (Sigma) and DOX 1 μM. ATP-γ-S 10 μM (Tocris) and Sodium Polyoxotungstate POM-1 (100 μM, Tocris) were used for creating ATP rich cell environment.

4.17 IMMUNOHISTOCHEMISTRY (IHC) AND IMMUNOCYTOCHEMISTRY (ICC) (II, III)

Lung tissues from two unrelated IPAH patients and two control patients, without previous history of pulmonary disease, were used for IHC (used primary antibodies are presented in table 3). The IHC-staining was done with ImmPRESS HRP polymer detection kit (Vector laboratories, Peterborough, UK)(II) or with Biocare Medical IHC kits (Concord, CA, USA)(III), both according to manufacturer's instructions. After DAPI detection the slides were stained with hematoxyllin.

For ICC-staining, ECs were seeded into 4-well chamber slides (Thermo Scientific, Lab-Tek), $50x10^3$ cells per well, let to attach and recover o/n. Next day cells were fixed with 4% formaldehyde and treated with Triton X-100 for permeabilization. After permeabilization and washings, slides were blocked with antibody blocking solution at 37°C for 1h and primary antibody was added, after additional washings, and incubated over night at 4°C. Before and after addition of secondary antibody FITC-conjugated AffiniPure (Jackson ImmunoResearch Laboratories, Suffolk, UK) slides were washed three times. Cells were stained

with DAPI including Vectashield mounting medium (Vector Laboratories, Peterborough, UK). Evaluation was done under fluorescent microscope.

In study IV, the CD39 siRNA silenced and control cells were irradiated with 5 Gy to induce DNA double strand breaks. After this the cells were left to recover for 4h in cell incubator before the cells were fixed and probed. The γH2AX positive foci were calculated with imageJ-software from single cells with 50 cells per condition.

4.18 STATISTICAL ANALYSES (I–IV)

Majority of the results are presented as column bars (mean ± SEM). Statistical analyses were done with GraphPad Prism™ software (La Jolla, CA, USA). For comparision between two groups we used unpaired two-tailed t-tests or Mann-Whitney U tests. For the analysis of multiple groups we used analysis of variance (One-Way ANOVA) with Bonferroni correction. P values lower than 0.05, were considered statistically significant.

RESULTS AND DISCUSSION

5.1 ECs AND OTHER CELL TYPES MAINTAIN CERTAIN NANOMOLAR LEVELS OF EXTRACELLULAR ATP, ADP AND ADENOSINE (IV)

ATP release to the extracellular milieu is one of the key components of purinergic signaling. By using our novel H_2O_2-sensing method with AmplexRed reagent, together with an enzyme-coupled bioluminescence assay, we were able to detect nanomolar concentrations of ATP, ADP, AMP, adenosine, inosine, hypoxanthine, and pyrophosphate all at the same time. The extracellular concentration in non-stimulated ECs (HUVEC and HDMEC) and EAhy926 cells was kept at low nanomolar level (~2–5 nM). Interestingly, the cancer cell lines Jurkat, MDA-MB-231, and B16-F10 kept considerably higher concentration of extracellular nucleotides (up to 25 nM). A particularly interesting finding was that the relatively high ATP/adenosine ratio in Jurkat T-cells was opposite to the low ratio in ECs, which might provide some insight regarding their dominant metabolic pathways (Figure 9).

Lymphoid and ECs have been reported to display both ATP-regenerating/adenosine-deaminating and nucleotide-inactivating pathways (92); however, the lack of ecto-5'nucleotidase/CD73 in Jurkat cells could explain the differences in the extracellular adenosine pools (92). Yet, little is known about the cellular mechanisms for basal level maintenance of purines. It is thought that the nanomolar extracellular ATP could reflect the concentration of the pericellular phase, which is localized in close proximity to the outer cell membrane (6, 236, 237). Our results are in line with previous studies reporting ATP release from excitatory and non-excitatory cells (4, 238, 239).

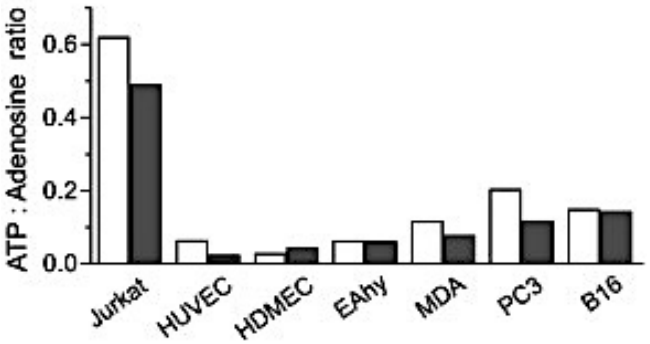

Figure 9. These histograms present the ATP/adenosine ratio at basal level in the bathing medium measured after 60-min incubation (white bars) and 120-min incubation (grey bars).

Next, induction of ATP secretion from HUVECs, EAhy926 cells and Jurkat T-cells was attempted, with the pro-inflammatory agents TNFα, interleukin-1β, dibutyryl cAMP, and

LPS as it is generally accepted concept that there is increased ATP levels at sites of inflammation. In tested cells, only the endotoxin LPS increased extracellular adenosine concentration by 50% but it failed to effect ATP concentrations. In addition, ECs appeared to preferably uptake adenosine via nucleoside transport NBT, while cancer cell lines use ecto-adenosine deaminase (ADA) to further metabolize the extracellular adenosine. Mechanical stimulation of ECs induced with harsh pipetting led to significant increase in extracellular ATP (Figure 10, unpublished results) but not adenosine concentrations. This increase might be caused by shear-stress induced ATP release (240) or due to convection of the cell-surrounding pericellular ATP pool into the external milieu.

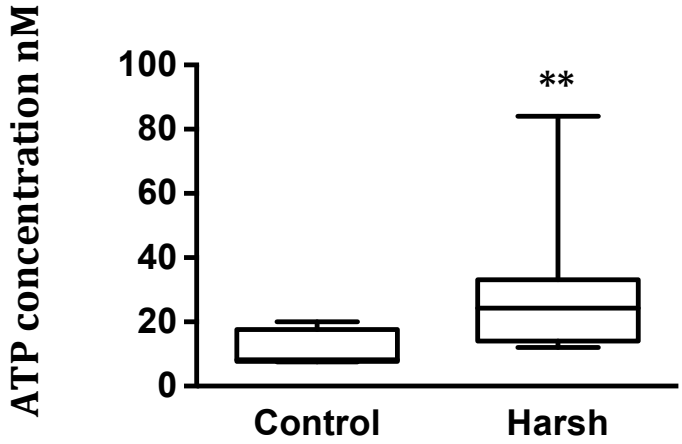

Figure 10. Harsh pipetting induces ATP release from vascular ECs. Harsh pipetting of the medium in three repetitions at the center of the wells with cultured EC (pooled results with MEC and HUVEC) increased the median of extracellular ATP level from 8.2 nM to 24.3 nM. 95% confidence box and whiskers, Mann-Whittney U-test was used for statistical analysis, p=0.0047.

5.2 NOVEL ATP, ADP, AMP, ADENOSINE, INOSINE, HYPOXANTHINE, AND PPi SENSING METHODS: RELIABILITY AND COMPARISON TO OTHER AVAILABLE METHODS (IV)

The fact that we obtained similar results from our fluorescence- and TLC-based enzyme-activity assay suggests that our new method works well. Even though the luciferin/luciferase method is well established for sensing ATP levels in various experimental settings, the method needs to be combined with particular mixtures of enzymes to allow broader substrate specificity, such as detection of ADP and PPi (169, 233, 236, 238). Similarly, AMP, adenosine, inosine, and hypoxanthine levels were measured with the use of specific enzyme mixtures, which sequentially convert AMP to H_2O_2. The end product H_2O_2 can be subsequently quantified with electrochemical (241), fluorometric (242), or chemiluminescence (243) based methods.

Other methods to detect purine concentrations include cell-based approaches (244, 245), HPLC analysis (246), fluorescent 1,N^6-ethenoadenine derivate compounds (247), atomic force microscopy (248), silicon nanowire devices (249), microscopic analysis of fluorescence in cells (250), and PPi detection by fluorescent or colorimetric assays (251). However, the main advantage of our approach is that we were able to detect ATP and all of its metabolites down to H_2O_2 from the same aqueous samples without any additional sample manipulation maneuvers. In addition, no sophisticated equipment is required for the method presented here except a fluorescence/luminescence detector.

5.3 ENDOTHELIAL CELLS DO NOT DISPLAY ECTO-NPP ACTIVITY (IV)

Autoradiographic TLC imaging and basal level measurements clearly demonstrated that cancer cells but not ECs are able to convert ATP into AMP and PPi. Our results show that metastatic cells (PC3 and MDA-MB-231) have both NTPDase and NPP activity in contrast to vascular ECs, which have been reported to have only NTPDase1/CD39 mediated ATPase activity (91, 169). In cancer research, the ATP-PPi axis has drawn very little attention as a potential regulator of cancer-cell migration and invasion. However, synthetic analogues of PPi called bisphosphonates have been used to treat metastatic cancers in bone (252). The current theory is that these effects are mediated through mevalonate/cholesterol biosynthesis inhibition or through other intracellular ATP-dependent pathways. Still, since some bisphosphonates are able to substitute PPi and form unhydrolysable ATP. Some of these anti-tumor effects could be caused by interference with the extracellular PPi homeostasis (253).

5.4 PURINE HOMEOSTASIS MEASURED IN THE HUMAN UMBILICAL VEIN (IV)

We tested whether the purine-sensing method (chapter 5.2) could be applied for evaluating purine homeostasis in tissues by using human umbilical cords. The medium collected from the washed umbilical vein had a very high hypoxanthine concentration (~3 μM) and nanomolar levels of adenosine and inosine. ATP and ADP levels were below the detection limit but some PPi (10 nM) was detected in the collected medium. The 20 min incubation with 2 μM ATP or adenosine failed to shift the spectrum of measured metabolites, which indicates that basal levels of purines are kept constant with efficient turnover of the released purines.

We believe that the luminal EC lining is the main regulator of the measured intravenous purine homeostasis and our results are fairly similar to those obtained from cultured HUVECs. The high nanomolar levels of adenosine and low ATP levels are consistent with the concept that adenosine is important in the maintenance of endothelial-barrier function as opposed to ATP, which may induce vessel leakiness.

5.5 DOWNREGULATED ECTO-ENZYME ACTIVITIES INDUCE CELL PROLIFERATION IN VVECS (I)

Expansion of the vasa vasorum network in chronically hypoxic calves has been previously described (230). Here, we focus on the role of endothelial ecto-enzymes in these conditions. We assayed specific ecto-nucleotidase and kinase activities with TLC using saturating concentrations of appropriate nucleotide substrates.

The hydrolyzing activities of CD73 and CD39 were decreased in chronically hypoxic VVECs. AMP hydrolysis by CD73 was decreased by approximately 80% ($p<0.05$) and CD39-mediated ADP (50% reduction, $p<0.05$) and ATP (~25% reduction, n.s.) hydrolysis rates were downregulated as well (Figure 11A). There were no changes, however, in the activities of the enzymes of the ATP-generating pathway (AdK and NDPK). As a consequence, the chronically hypoxic VVECS kept considerably higher basal extracellular levels of ATP (~60%, $p<0.05$) and ADP (~65%, $p<0.05$), as measured 1h after media change (Figure 11B). It should be considered that higher micromolar ATP concentrations could be maintained in the pericellular space, while only a fraction is detected in the bulk medium (2).

It seems that vascular ECs react to various stress conditions such as chronic hypoxia, but also to inflammation and oxidative stress, by attenuating their NTPDase1/CD39 activity (141, 254).

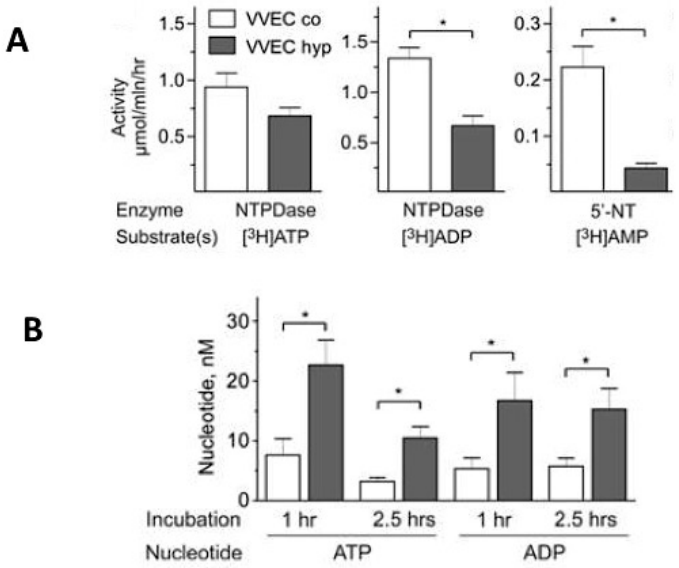

Figure 11. A. ATP, ADP, and AMP hydrolyzing ecto-enzyme activities in control and chronically hypoxic VVEC. **B.** Extracellular ATP and ADP concentrations as measured at 1 hour and again 2.5 hours after media change.

Both ATP and ADP were able to induce VVEC proliferation in [H^3]thymidine incorporation assay, whilst, the cells from chronically hypoxic calves were significantly more proliferative at lower ATP and ADP concentrations (10^{-6}M), and control cells isolated from normoxic animals needed 10 times higher ATP and 100 times higher ADP concentrations for activation. Together the results imply that even a slightly elevated extracellular ATP concentration is sufficient to activate P2 receptors. This might lead to the development of a pro-angiogenic and hyperproliferative EC phenotype, which in turn could lead to pathological vascular remodeling, such as observed in PAH. Importantly, this high ATP sensitivity is a characteristic feature of ECs in small vessels since ECs from systemic vessels have much weaker responses to ATP and significantly higher NTPDase activities (255).

Surprisingly, the changes in nucleotidase activities were not mediated by changes in their expression levels. No significant changes were seen in CD39 or CD73 mRNA expression levels in chronically hypoxic cells compared to control cells. Also CD39 protein expression was constant in both groups. Unfortunately we did not find a working antibody against bovine CD73 for protein-level detection.

In addition to possible changes in protein-expression levels, the enzymatic activities of CD39 and CD73 can be post-translationally modified. Both ATP and ADP, which were accumulated in hypoxic animal cells, are known to inhibit the CD73 AMP hydrolysing activity (91). In addition, adenosine further activates CD73 activity, while a decrease in adenosine levels could inhibit CD73 even further (256). CD39 enzymatic activity can be upregulated by the formation of multimers, or downregulated through monomerization or oxidative inactivation(254, 257)

5.6 DOWNREGULATED EC CD39 ACTIVITY IN PULMONARY ENDOTHELIUM COULD LEAD TO EC AND SMC DYSFUNCTION (II)

Even though CD39 activity can be post-translationally modified, its activity is mainly regulated by changes in expression(258)We used immunohistochemistry to visualize the expression of CD39 in lung endothelium from three unrelated iPAH patients and unaffected controls. We observed that the expression of CD39 was markedly reduced in the intact endothelium of all remodelled vessels of the iPAH patients compared to the healthy arteries of the control patients (Figure 12).

We used ECs isolated from two IPAH patients to evaluate CD39 expression levels. CD39 protein expression level was significantly decreased in iPAH ECs in these analyses. The protein level down-regulation of CD39 was in line with the measured 57% decrease in specific ATP hydrolysing activity. Furthermore, recovery to basal level after 2 μM ATP addition was significantly attenuated (80%) in iPAH ECs.

To confirm our findings we used the rat model of PAH and utilized our magnetic-bead based *in vivo* profiling strategy to measure CD39 activity without cell culturing. The results fit well together with our human studies, since the ECs from MCT-injected animals had significantly decreased CD39 ATPase enzymatic activity (45%) five days after MCT injection.

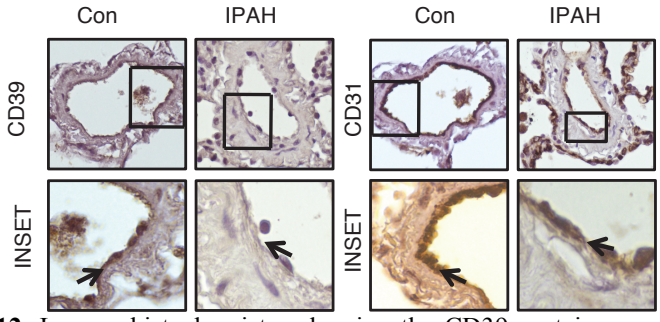

Figure 12. Immunohistochemistry showing the CD39 protein expression is decreased in remodelled vessel from a PAH patient compared to a healthy control while the endothelial cell layer remains intact based on the EC marker CD31 staining.

To study the CD39 deficient EC phenotype in control cells we utilized siRNA techniques to reduce CD39 expression. In our studies, EC downregulation of CD39 alone led to the activation of ERK1/2, which suggests that the loss of CD39 leads to a niche with increased ATP, capable of activating the ERK1/2 pathway. These results are in line with a previous report showing that ATP can activate the ERK1/2 pathway in endothelial cells (259).

CD39 down-regulation with siRNA significantly increased cell proliferation by 20% in our cell-count experiments. This phenotype change was accompanied with increased resistance to apoptosis as measured by caspase 3/7 activation (25% more resistant). The cells made CD39 deficient with siRNA also showed significantly higher metabolic activity in XTT-assays (30% increase), when compared to control ECs. Similarly, 2 μM ATP alone was able to increase resistance to serum starvation induced caspase 3/7 activation (30% more resistant) and increase metabolic activity in XTT assay (100% increase), when compared to control ECs. Interestingly, IPAH ECs demonstrated no response at all to the added 2 μM ATP in similar caspase 3/7 and NAPDH assays.

In conclusion, the loss-of CD39 ATPase activity is similar to the phenotypic switch observed in pulmonary ECs during PAH pathogenesis (217) which led to an apoptosis-resistant and hyperproliferative phenotype.

We were interested to see if a small ATP gradient from CD39-deficient ECs could influence the surrounding SMCs. We tested this by initiating a co-culture system. We observed a significant 20% increase in SMC migration through a porous membrane, towards the CD39-deficient ECs, when compared to control ECs. Furthermore, 2 or 10 μM ATP alone was able to increase the SMC migration.

In line with previously published studies (173, 174), 2 μM ATP induced a 50% increase in SMC viability in XTT assays. These identified effects on SMC are mediated through P2 receptor activation, but they might be attributable also by adenosine receptor inhibition caused by decreased adenosine levels in the CD39-deficient cell co-culture system. Since adenosine inhibits SMC proliferation and promotes SMC apoptosis, adenosine might be an important modulator of intima thickness (260).

5.7 ADENOSINERGIC REGULATION OF THE VASCULAR BARRIER IN DYSFUNCTIONAL VVECs FROM CHORICALLY HYPOXIC CALVES (I)

It has been well established that hypoxia induces vascular leakage (261), which can be regulated through adenosinergic signaling (262). The role of adenosinergic signaling under chronic hypoxia in vascular leakage in VVECs has not been studied previously, however. EC monolayer permeability was studied by measuring the flux of FITC-dextran through the cell layer.

Cells from chronically hypoxic animals had significantly compromised barrier function as it took only 15 min to reach 50% of maximal fluorescence on the other side of cell mono-

layer while in control cells this took 40 min to develop. Various pre-treatments such as NECA (non-selective adenosine receptor agonist), CPCA (A_2- selective agonist), ATP and AMP enhanced the barrier function of control cells, but were unable to improve the barrier function in cells isolated from chronically hypoxic calves.

A_2 adenosine receptors are linked to a stimulatory G-protein receptor, which increases intracellular cyclic AMP (cAMP) upon activation. In addition, cAMP has been shown to be involved in pulmonary endothelial vascular barrier function maintenance (263). This led us to hypothesize that cAMP production, upon adenosine receptor ligation, could be ineffective.

Surprisingly, no differences were seen in the intracellular cAMP levels after A_2 receptor stimulation, between cells from chronically hypoxic animals and control cells. These results suggest that irresponsiveness to adenosinergic EC barrier function enhancement is not because of dysfunctional cAMP generation after A_2 receptor stimulation. One possibility is that the disturbed adenosine homeostasis due to CD73 and CD39 downregulation will adversely affect the VVEC barrier function in cells from chronically hypoxic calves via an unknown mechanism. These effects cannot be compensated through adenosine receptor A_2 activation.

The results presented here are in agreement with the increased vascular permeability that has been reported previously in the lungs of $CD73^{-/-}$ mice (264) and numerous studies that have shown the importance of CD73 in the control of EC barrier function and leukocyte migration through the EC layer (74, 81, 256, 265, 266). In chronic hypoxia, vascular-EC barrier function is dysfunctional in PAH, probably due to decreased BMPR2 expression (267), which could be an additional mechanism for adenosine-controlled vascular permeability. Moreover, it has been shown that hypoxia downregulates BMPR2 expression and thus affects to the BMPR2 mediated cell signaling (268), suggesting a common regulator for both CD73 and BMPR2 expression.

5.8 ATP MEDIATED EC ACTIVATION COULD BE DEPENDENT ON THE P2Y11 ATP RECEPTOR (II)

A screening was next made for a candidate purinergic receptor that could be important for the observed ATP-mediated effects and would prefer ATP as a substrate molecule. P2Y11 receptor was the most downregulated purinergic receptor by re-evaluating data from a previously published microarray study where bone morphogenetic protein receptor 2 (BMPR2), a key receptor in PAH, was downregulated with siRNA on healthy human pulmonary ECs (231). As BMPR2 is known to regulate pulmonary EC survival (269) we hypothesized that P2Y11, which only recognizes ATP as a substrate (270, 271), could be important for ATP mediated responses. The function of P2Y11 receptor in EC biology is fairly unknown and only few papers exist. In one of these these papers the P2Y11 receptor has been linked to cell-cycle arrest and cell death with high ATP concentrations (50-100 μM) in human umbilical cord ECs and aortic ECs (272).

First we confirmed that the P2Y11 receptor is expressed in the lung endothelium by lung-section IHC-staining and by quantifying HMVEC and iPAH-patient P2Y11 mRNA and protein-expression levels. At mRNA levels the P2Y11 receptor was similarly expressed in both control and iPAH-patient derived cells. However, there was a quantified 50% up-regulation in the protein expression level of four iPAH patients. To study the P2Y11 deficient EC phenotype we used siRNAs to down-regulate P2Y11 expression in ECs.

In our experiments, P2Y11 down-regulation leads to a significant 20% decrease in proliferation with cell-count experiment and significantly increased caspase 3/7 activation under serum starvation (60% more activation). Furthermore, P2Y11-deficient ECs also had a 70% decreased metabolic rate compared to control siRNA-treated ECs in the XTT assay. Interestingly, the P2Y11 siRNA treatment also sensitized the iPAH cells to serum-starvation-induced caspase 3/7 activation by 35% and decreased the cells metabolic rate by 50%.

P2Y11 is clearly an important mediator of ATP-induced cell viability since P2Y11-deficient healthy ECs were completely resistant to ATP-mediated survival and viability responses, while control non-targeting siRNA-treated ECs responded to ATP treatment. In line with these results, the P2Y11 receptor agonist β-NAD induced caspase 3/7 activation under serum starvation and increased the metabolic rate in healthy ECs, as seen with ATP treatment.

Activation of the G-protein linked P2Y11 receptor can induce both calcium mobilization and cAMP pathways, which is a unique feature of the P2Y11 receptor (270). It would seem, however, that the enhanced cell survival would be mediated by the cAMP pathway (unpublished data). A sustained ATP gradient created by the down-regulation of CD39 could mediate the observed inactivity to ATP signaling in iPAH and CD39 knock-down cells through saturation of P2 receptors, such as P2Y11.

One plausible explanation for the fact that iPAH and control cells had similar P2Y11 mRNA expression level, but a 50% difference in protein expression, could be that the turnover rate of P2Y11 receptor is slower in iPAH cells. This could also explain why P2Y11 mRNA silencing led to less dramatic results in iPAH cells than with control cells. The P2Y11 receptor has not been identified in rodents, which makes it challenging to study in *in vivo* conditions. Even so, the P2Y11 receptor might account at least partly for the major differences between rodent and human PAH.

5.9 APELIN-13 PEPTIDE CAN INCREASE THE CELL BOUND CD39 ATPASE ACTIVITY (II)

Apelin peptide, which is endogenously expressed and an important regulator of vascular homeostasis, could have therapeutical use in PAH, based on results from hypoxia mouse and MCT rat models of PAH (231, 273). It is poorly understood, however, how apelin delivers its therapeutic effects. We hypothesized that the effects of apelin could be linked to

CD39 activity since increased CD39 activity could be a beneficial way to inhibit further vascular remodeling. Interestingly, when we incubated ECs with Apelin-13 peptide overnight, CD39 activity increased in a concentration-dependent manner. This CD39 activity upregulation could only be detected with cell bound forms. The peptide failed to stimulate soluble forms of ATPases, such as Apyrase or other soluble forms from serum (unpublished data). The highest activation of CD39 was measured with 1 μM apelin, which resulted in a 300% increase in CD39 activity.

Incubation with apelin had no effect on EC CD39 mRNA expression, which suggests that apelin would modulate the CD39 activity at a post-translational level. This is most likely achieved by enhancing multimer formation or by changing the catalytic centre of CD39. Apelin failed to activate CD73 activity in a TLC-based assay even though we measured a 29% increase in the level of AMP breakdown product adenosine in in EC medium was measured by a fluorescence-based method after an hour of exposure to apelin and ATP. This result indicates a difference in ATP hydrolysis efficiency since CD39 down-regulation can also lead to decreased adenosine levels.

The difference in adenosine production caused by apelin could be crucial for the worsening of PAH pathobiology, since it is well appreciated that adenosine enhances vascular-barrier function and inhibits inflammation (175). In addition, suppression of CD39 can lead to the accumulation of ADP, which can activate platelets at injury sites, as well as induce inflammation(194, 274). Furthermore, increased thrombosis and decreased adenosine levels in plasma have been recognized in patients with severe PAH (190, 195).

Even a 30-minute incubation time with apelin was sufficent to increase the surface CD39 activity on ECs by 60% in healthy cells and by 17% in cells from iPAH patient. The difference in activation levels reflected the difference in expression levels since there is less CD39 on iPAH cells, for example for cluster formation. In addition, apelin silencing with siRNA techniques also significantly decreased CD39 mRNA expression levels (37% decreased expression).

In the same EC–SMC co-culture system, as we previously used, the administration of 1 μM apelin to the ECs caused a 40% reduction in the migration of SMCs when compared to control conditions. This effect is likely to be due to a decreased ATP niche in SMC proximity. Interestingly, others have shown that blocking of connexin 43 hemichannels also inhibits vascular remodeling by reducing the extracellular ATP niche (26) that could reduce SMC proliferation and migration. In addition, we repeated the rat-MCT experiment with apelin treatment in order to evaluate whether the administration of apelin could increase the activity of CD39 ATPase *in vivo*.

Apelin treatment did not affect CD39 ATPase activity in healthy animals. In the rat model of PAH, where the rats received MCT injection together with the apelin treatment, the ATPase activity was increased by 20%, indicating that apelin can also increase CD39 activity *in vivo*. Apelin treatment has been shown to be beneficial in MCT and hypoxia mouse models of PAH (275, 276) but the mechanism is not fully understood. The expression level

of apelin receptor (APJ) in pulmonary ECs was below detection in our experiments, both in control and IPAH ECs, similar to a previously reported study (231). Based on the obtained results, the molecular mechanism of apelin treatments could be mediated by increase in CD39 ATPase activity, which could modify the extracellular purinergic homeostasis to more favorable direction.

5.10 EXTRACELLULAR ATP PROTECTS ENDOTHELIAL BUT NOT CANCER CELLS AGAINST IRRADIATION INDUCED OR CHEMICALLY INDUCED DNA DAMAGE (III)

Because double-strand repairs are done without a template, the process is more prone to errors (277). After a double-strand break, the close-by H2A histone variant H2AX is quickly phosphorylated. This phosphorylated form (γH2AX) is widely used as a biomarker for double strand breaks (278). The γH2AX functions as an alert signal for the repair machinery and it has a substantial role in DNA-stability maintenance (278). Several kinases from the phosphatidyl inositol 3-kinase family, such as DNA-dependent protein kinases (DNA-PK), ataxia telangiectasiamutated (ATM) kinases, and ATR (ATM and Rad-3 related) kinases, are activated upon DNA double-strand breaks (279). These kinases activate several components required for DNA repair and to stop DNA replication until the breaks have been repaired (277).

Stable ATP analogue ATP-γ-S pretreatment significantly reduced the amount of DNA damage in terms of expression level of the early DNA damage marker γH2AX. These results were consistent in all measured experimental settings in which we used irradiation or MMS, which alkylates bases in DNA, to induce DNA damage in cells.

CD39 activity was next silenced with siRNA, similar to study II, in order to create an ATP-rich niche in proximity to the cells. Surprisingly, the basal level of DNA damage was also decreased in CD39-deficient cells and the difference was enforced after irradiation treatment. In the CD39 downregulated cells, γH2AX protein expression was 50% lower 45 min after irradiation, and 75% lower 4h after irradiation. These results were also visualized with immunocytochemistry in addition to the quantification made from western immunoblots (Figure 13). The DNA damage marker γH2AX protein expression was also decreased in CD39-downregulated cells after doxorubicin (DOX) treatment. Similar results, as with CD39 siRNA-treated cells, were obtained with POM-1, which is a chemical inhibitor of CD39, treated cells. In addition, we used p53, a classical marker for DNA damage, to confirm our findings.

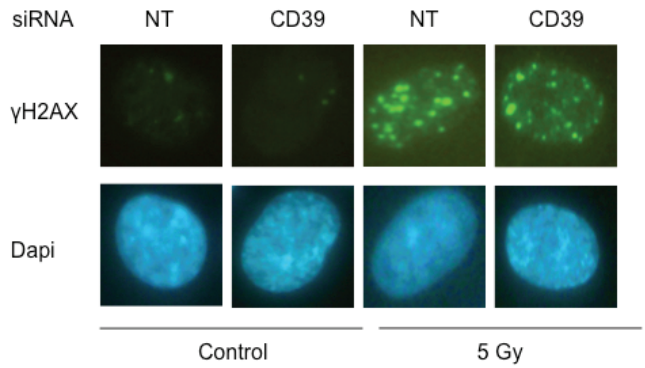

Figure 13. The nuclei γH2AX immunocytochemistry from control and CD39 deficient ECs shows that CD39-deficient cells are less prone to irradiation-induced DNA double-strand breaks. Cells were stained at the basal level and after 5 Gy irradiation.

In vivo rat models were next chosen to expand on the work already conducted *in vitro*. Animals pre-treated with POM-1 were given MCT, a drug widely used in PAH studies, which also induces DNA damage (280) or DOX. In the animals pre-treated with POM-1 we saw significantly decreased expression levels of γH2AX measured in heart and lung samples. These results were consistent between western blots made from whole tissue lysates and immunohistochemistry assays.

Whether ATP would also protect cancer cell lines against DNA damage, which would prevent the therapeutic use of ATP against DNA damage in healthy cells, was next tested. For this, human leukemia (K562) and lymphoma (SUDHL4) cell lines were employed, where the DNA damage was induced with DOX, a drug that can be used to treat both malignancies(281, 282)Reassuringly, the results clearly demonstrated that POM-1 pre-treatment did not decrease the γH2AX protein expression level in either of the used cell lines. In addition, 24h DOX treatment induced a significant caspase 3/7 activation in both studied cancer cell types. Pretreatment with POM-1 was unable to decrease the caspase 3/7 activation but actually increased it in K562 cells. This could be because of different downstream ATP receptor signaling compared to ECs or due to the fact that cancer cells have high NPP activity, while ECs demonstrate no NPP activity, which POM-1 does not inhibit.

CONCLUSIONS AND FUTURE PERSPECTIVES

❖ This dissertation describes a novel method to detect all ATP-derived nucleotide and nucleoside down-stream metabolites with the combined use of enzyme coupled bioluminescent and fluorometric assays. With the use of this method, vascular ECs, leukocytes, and tumor cells maintain basal levels of extracellular ATP, ADP, AMP, adenosine, and PPi at certain characteristic nanomolar levels. In addition, we have shown that this method can be applied to study purine-converting ecto-enzyme pathways.

❖ NTPDase1/CD39 and ecto-5´-nucleotidase/CD73 activities were decreased in ECs isolated from chronically hypoxic calves. These cells were characterized by higher extracellular levels of ATP and reduced levels of adenosine compared to control cells. These shifts in purine homeostasis may contribute to abnormal cell proliferation and barrier function, and favoured angiogenic processes.

❖ This study on ATP-mediated signaling in PAH pathogenesis revealed a significant suppression of NTPDase1/CD39 in end-stage human IPAH patients and in a rat model of PAH. Based on these results, loss of CD39 activity lead to an increased concentration of extracellular ATP, which could modulate the cell phenotype of both ECs and SMCs.

❖ Diminished ecto-enzyme activities led to an increase in extracellular nucleotide levels. Even a small (2-10 μM) ATP concentration was sufficent to induce pulmonary EC viability, proliferation and pulmonary SMC proliferation and migration.

❖ CD39 deficient and ATP pre-treated ECs were more resistant to irradiation or chemotherapy-induced DNA damage. However, it seemed that cancer cells were not protected after CD39 inhibition. This makes the use of ATP mediated signaling clinically feasible for possible future applications.

❖ Altogether, our results suggested that sustained CD39 expression could be protective against vascular remodeling in PAH, but also during chronic hypoxia. This hypothesis was further supported by a recently published study on angioplasty-induced vascular injury, where over-expression of CD39 reduced the development of neointima (283). However, it seemed that ATP preconditioning before the initial vascular injury could be benefial in preventing vascular remodeling in selected cases. This disparency could be explained with acute versus chronic exposure to elevated ATP. In other words short term ATP activation seems to be beneficial for EC function but sustained activation may lead to pathological conditions.

For future perspectives, it would be intriguing to study the mechanism behind apelin modulated CD39 activity. Moreover, it would be interesting to see which of the apelin peptides, and at what concentration, would be the most efficient in activating CD39. Altogether, our results reveal that alterations in purinergic homeostasis could significantly contribute to pulmonary vascular remodeling in PAH. We also showed that these alterations could be

closely linked to other signaling pathways and molecules previously associated with PAH pathogenesis. Moreover, we have demonstrated that the purinergic pathway represents an attractive target for future therapies to treat PAH.

The in-depth downstream signaling pathways of ATP in endothelial cells of PAH patients remain unknown. We have some preliminary results suggesting that CD39 expression could be linked to BMPR2, the hallmark of PAH, but this finding would need further investigation. Moreover, it would be interesting to study how the blockage of ATP release by targeting vascular Cx43 channels in ECs and SMCs would affect the observed pathological vascular remodeling.

The mechanism of ATP-induced DNA repair remains unknown. We can speculate it might be linked to the function of topoisomerases responsible for the NHEJ or alternatively, to BMPR2 mediated signaling that has been shown to protect cells from irradiation and chemically induced DNA damage (219). The efficient use of ATP targeted cell protection in the clinics would still require additional clarification of the receptors and downstream signaling pathways.

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Helsinki, October 2015

Mikko Helenius

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