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# ATP release from vascular endothelia occurs through connexin 43 and is attenuated during hypoxia

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# 1. ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-Binding Cassette |
| $AdoRA_1$ | Adenosine Receptor $A_1$ |
| $AdoRA_{2A}$ | Adenosine Receptor $A_{2A}$ |
| $AdoRA_{2B}$ | Adenosine Receptor $A_{2B}$ |
| $AdoRA_3$ | Adenosine Receptor $A_3$ |
| ADP | Adenosine Diphosphate |
| AMP | Adenosine Monophosphate |
| ATP | Adenosine Triphosphate |
| BFA | Brefeldin A |
| bHLH | Basic Helix-Loop-Helix |
| BIM | Bisinodylmaleimide |
| BMK 1 | Big Mitogen Activated Protein Kinase |
| BSA | Bovine Serum Albumine |
| cAMP | Cyclic Adenosine Monophosphate |
| CAP37 | Cationic Antimicrobial Protein |
| CBP | CREB- Binding Protein |
| cDNA | Complementary Desoxyribonucleic Acid |
| CD39 | Ecto-Apyrase |
| CD73 | Ecto-5´-Nucleotidase |
| CNT | Concentrative Nucleoside Transporter |
| Chr | Chromosom |
| Cx | Connexin |
| DNA | Desoxyribonucleic Acid |

| | |
|---|---|
| ENT | Equiliberative Nucleoside Transporter |
| ERK-1 | Extracellular Signal Regulated Kinase |
| FMLP | N-Formyl-Metionyl-Leucine-Phenylaline |
| 18αGA | 18α-Glycyrrhetinic Acid |
| GHSR | Growth Hormone Secretagogue Receptor |
| GJ | Gap Junction |
| HBP | Heparin-Binding Protein |
| HBSS | Hanks´ Balanced Salt Solution |
| HEPES | 2-(4-(2-Hydroxyethyl)1-Piperazinyl)-Ethansulfonsäure |
| HEV | High Endothelial Venules |
| HMEC-1 | Human Microvascular Endothelial Cells |
| HIF | Hypoxia Inducible Factor |
| HPC | Intermittend Hypoxia |
| HPLC | High Performance Liquid Chromatography |
| HUVEC | Human Umbilical Vein Cells |
| Ig | Immunglobuline |
| IP | Ischemic Preconditioning |
| $IP_3$ | Inositol-Triphosphate |
| JAM | Junctional Adhesion Molecule |
| LDH | Lactat Dehydrogenase |
| MAPK | Mitogen-Activated Protein Kinase |
| MEF2C | Myocyte Enhancer Factor |
| mGluR | Metabotropic Glutamate Receptor |
| MPO | Myeloperoxidase |
| mRNA | Messenger Ribonucleic Acid |
| NDS | Neutrophil-Derived Secretagogue |

| | |
|---|---|
| NTPDase | Ecto-Nucleoside-Triphosphate Diphosphohydralase |
| ODD | Oxygen Dependent Degradation Domain |
| Panx | Pannexin |
| PAS | Per-ARNT-Sim |
| PBS | Phosphate Buffered Saline |
| PHD 1 | Prolyl-Hydroxylase Domain 1 |
| PHD 2 | Prolyl-Hydroxylase Domain 2 |
| PHD 3 | Prolyl-Hydroxylase Domain 3 |
| PKA | Protein Kinase A |
| PKC | Phosphokinase C |
| PMN | Polymorphonuclear Leukocytes |
| POM | Polyoxometalate |
| PVDF | Polyvinylidene Fluoride |
| pVHL | Von Hippel-Lindau-Protein |
| P2X | Purinergic Receptor 2X |
| P2Y | Purinergic Receptor 2Y |
| PX | Purinergic Receptor X |
| RNA | Ribonucleic Acid |
| RT-PCR | Real-Time Polymerase Chain Reaction |
| SCR | Scrambled |
| SDS-PAGE | Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis |
| siRNA | Small Interfering Ribonucleic Acid |
| TEM | Transendothelial Migration |
| TPA | 12-O-Tetradeconyl-Phorbol-13-Acetat |
| UDP | Uridine Diphosphate |

| | |
|---|---|
| UPR | Untranslated Region |
| UTP | Uridine Triphosphate |
| VASP | Vasodilatator-Stimulated Phosphoprotein |
| VEGF | Vascular Endothelial Growth Factor |

# 2. TABLE OF CONTENTS

# 3. INTRODUCTION

## 3.1. Vascular barrier

### 3.1.1. Structural and functional elements of the vascular barrier

The predominant barrier (~90%) to movement of macromolecules across a blood vessel wall is presented by the endothelium (2, 3). Passage of macromolecules across a cellular monolayer can occur via either a paracellular route (i.e., between cells) or a transcellular route (i.e., through cells). In non-pathologic endothelium, macromolecules such as albumin (molecular weight ~66 kD) appear to cross the cell monolayer by passing between adjacent endothelial cells (i.e., paracellular) although some degree of transcellular passage may also occur (4, 5). Endothelial permeability is determined by cytoskeletal mechanisms that regulate lateral membrane intercellular junctions (6, 7). Tight junctions, also known as zona occludens, comprise one type of intercellular junction. Transmembrane proteins found within this region which function to regulate paracellular passage of macromolecules include the proteins occludin, and members of the junctional adhesion molecule (JAM) and claudin families of proteins (8). Tight junctions form narrow, cell-to-cell contacts with adjacent cells and comprise the predominant barrier to transit of macromolecules between adjacent endothelial cells (9). Endothelial macromolecular permeability is inversely related to macromolecule size. Permeability is also dependent on the tissue of origin. For example, endothelial cells in the cerebral circulation (i.e., blood-brain barrier) demonstrate an exceptionally low permeability (10, 11). Endothelial permeability may increase markedly upon exposure to a variety of inflammatory compounds (e.g.,

histamine, thrombin, reactive oxygen species, leukotrienes, bacterial endotoxins) or adverse conditions (e.g., hypoxia, ischemia) (2, 12). Reversible increases in endothelial permeability are produced by administration of cytochalasin or other agents that disrupt cytoskeletal microfilaments (2, 13). Likewise, increases in endothelial permeability are accompanied by disruption of peripheral actin microfilaments and formation of gaps between adjacent endothelial cells (2, 13). Administration of compounds that decrease endothelial permeability result in an irregular endothelial cell contour, greater convolution of cell margins, closer cell-to-cell contact, and increased surface area and cell perimeter (13). These changes in cell morphology are accompanied by a loss of F-actin in stress fibers, “ruffling” of dense peripheral bands of F-actin, and increase in the polymerized actin pool without significant changes in total F-actin endothelial cell content (6, 7). Interestingly, these changes in intracellular actin are similar to those observed during PMN transendothelial migration (14). By comparison, thrombin-induced increases in permeability result in a centralization (and peripheral loss) of F-actin. Both of these changes (permeability and F-actin distribution) are inhibited by isoproterenol (15). Phallacidin, an F-actin-stabilizing compound, also markedly attenuates thrombin-induced increases in permeability and accompanying morphologic changes.

In addition to the above components of the vascular barrier, the glycocalyx may play a role in determining movements of fluid and macromolecules across the endothelium. The endothelial glycocalyx is a dynamic extracellular matrix composed of cell surface proteoglycans, glycoproteins, and adsorbed serum proteins, implicated in the regulation and modulation of capillary tube hematocrit, permeability, and hemostasis (16). As such, increased paracellular

permeability of such molecules as water, albumin and hydroxyethyl starch can be observed following experimental degradation of the functional components of the glycocalyx (17), and functional components of this glycocalyx may be dynamically regulated by endogenous mediators such as adenosine (18).

### 3.1.2. Barrier protective pathways

Acute increases in vascular permeability to macromolecules closely coincides with tissue injury of many etiologies, and can result in fluid loss, edema, and organ dysfunction (2, 19, 20). Previous studies have indicated that extracellular nucleotide metabolites may function as an endogenous protective mechanism during hypoxia and ischemia (21-23). One important factor may be increased production of endogenous adenosine, a naturally occurring anti-inflammatory agent (23-25). Several lines of evidence support this assertion. First, adenosine receptors are widely expressed on target cell types as diverse as leukocytes, vascular endothelia, and mucosal epithelia and have been studied for their capacity to modulate inflammation (26). Second, murine models of inflammation provide evidence for adenosine receptor signaling as a mechanism for regulating inflammatory responses in vivo. For example, mice deficient in the $A_{2A}$-adenosine receptor ($AdoRA_{2A}$) show increased inflammation-associated tissue damage (27). Third, hypoxia is a common feature of inflamed tissues (28) and is accompanied by significantly increased levels of adenosine (29-31). At present, the exact source of adenosine is not well defined, but likely results from a combination of increased intracellular metabolism and amplified extracellular phosphohydrolysis of adenine nucleotides via surface ecto-nucleotidases.

The vascular endothelium is the primary interface between a hypoxic insult and the surrounding tissues. At the same time, the endothelium is central to the orchestration of leukocyte trafficking in response to chemotactic stimuli. This critical anatomic location places vascular endothelial cells in an ideal position to coordinate extracellular metabolic events important to endogenous anti-inflammatory responses. Recently a neutrophil-endothelial cell crosstalk pathway was identified that is coordinated by hypoxia (32). This pathway utilizes extracellular nucleotide substrates, liberated from different cell types. Extracellular ATP release has been shown from endothelial cells, particularly under sheer conditions (33) or during hypoxia (34). In addition, fMLP activated neutrophils can release ATP, however the exact mechanism through that these cells release ATP isn´t cleared obviously (35, 36). Activated platelets comprise an additional source for extracellular adenine nucleotides (37, 38). The role of endothelial CD39 (Ecto-apyrase, conversion of ATP/ADP to AMP) has been viewed as a protective, thromboregulatory mechanism for limiting the size of the hemostatic plug (38, 39). Metabolism of adenine nucleotides derived from activated platelets is crucial in limiting excessive platelet aggregation and thrombus formation (40, 41). Similarly, excessive platelet accumulation and recruitment can be treated with the use of soluble forms of CD39 (42, 43). Moreover, a thromboregulatory role could be demonstrated in a model of stroke, where cd39-null mice showed increased sizes of infarction that could be reduced by treatment with soluble CD39 (44). Surprisingly, targeted disruption of CD39 resulted in prolonged bleeding and increased vascular leak and fibrin deposition in hypoxemia (45), suggesting a dual role for ATP metabolism by CD39 in modulating hemostasis and thrombotic reactions. Moreover, this observation may be related to an activation and desensitization of the

purinergic type $P2Y_1$ receptor. Activation of the $P_2Y_1$-platelet receptor appears to be crucial in the activation process of platelets. As such, $P2Y_1$ deficient mice exhibit signs of prolonged bleeding time and resistance to thromboembolism (46).

Extracellular ATP is readily converted on the endothelial surface to adenosine, due to the enzymatic function of CD39 (see later) and CD73 (5'-Ecto-nucleotidase, conversion of AMP to adenosine). Such adenosine binds to surface expressed PMN adenosine receptors to limit excessive accumulation of PMN within tissues, and as such, functions as a feedback loop to attenuate potential tissue injury (36). With regard to this latter point, it was recently shown that hypoxia coordinates both, transcriptional and metabolic control of the surface ecto-nucleotidases CD39 and CD73 (35, 47, 48), and as such, significantly amplifies the extracellular production of adenosine from adenine nucleotide precursors. Studies using cd39- and cd73-null animals showed that extracellular adenosine produced through adenine nucleotide metabolism during hypoxia is a potent anti-inflammatory signal for PMN in vitro and in vivo (36). These findings identify CD39 and CD73 as critical control points for endogenous adenosine generation and implicate this pathway as an innate mechanism to attenuate excessive tissue PMN accumulation (36).

In addition to a role in limiting excessive neutrophil tissue accumulation, CD39 and CD73 are also critical control points for vascular permeability. For the purpose of investigating overall organ vascular permeability, Evan's blue dye, which tightly binds to plasma albumin (49), was used. This model entails the quantification of formamide-extractable Evans blue (50) from tissues of mice as a readout for overall vascular permeability of different organs. In fact it was found that vascular permeability in tissues derived from animals subjected to

normobaric hypoxia (8% $O_2$/92% $N_2$) ranged from 2 - 4-fold more permanent to Evans blue than normoxic controls (35).

In order to identify the role of CD73 in vascular permeability, this model in mice was used that were administered with the CD73 inhibitor APCP or in mice following targeted deletion of CD73 (51). In fact, dramatic increase of hypoxia-elicited dysfunction of the vascular barrier in different organs (lung, heart, intestine, kidneys) was found following CD73 inhibition or deletion. Vascular leak associated with hypoxia was, at least in part, reversed by reconstitution with soluble 5'-nucleotidase and adenosine receptor agonists in the cd73-null mice. Histological examination of lungs from hypoxic $cd73^{-/-}$ revealed perivascular interstitial edema associated with inflammatory infiltrates surrounding larger pulmonary vessels (51). Taken together, these studies identify CD73 as a critical mediator of vascular permeability in vivo.

When measuring vascular permeability during hypoxia in mice with targeted deletion of CD39, similar increases in vascular barrier function could be observed in different organs (35). Taken together, these studies have identified adenosine generation of the hypoxic vasculature via nucleotide-phosphohydrolysis as a critical cellular strategy to generate adenosine and maintain vascular barrier function.

### 3.1.3. Barrier disruptive pathways

Macromolecule transit across blood vessels has evolved to be tightly controlled. Relatively low macromolecular permeability of blood vessels is essential for maintenance of a physiologically optimal equilibrium between intravascular and extravascular compartments (52, 53). Endothelial cells are primary targets

during episodes of infection, ischemic or traumatic injury, which all together can result in an altered barrier function. Disturbance of endothelial barrier during these disease states can lead to deleterious loss of fluids and plasma protein into the extravascular compartment. Such disturbances in endothelial barrier function are prominent in disorders such as shock and ischemia-reperfusion and contribute significantly to organ dysfunction (54-58).

Previous studies have indicated that activated PMN release a number of soluble mediators, which dynamically influence vascular permeability during transmigration. As such, PMN have been shown to liberate factors that can either disrupt or protect the endothelial barrier: For example, it was recently shown that activation of PMN through $\beta_2$ integrins elicits the release of soluble factor(s) which induce endothelial cytoskeletal rearrangement, gap formation and increased permeability (59). This PMN-derived permeabilizing factor was subsequently identified as heparin-binding protein (HBP, also called azurocidin or CAP37 (59), a member of the serprocidin family of cationic peptides (60). HBP, but not other neutrophil granule proteins (e.g. elastase, cathepsin G), was shown to induce $Ca^{2+}$-dependent cytoskeletal changes in cultured endothelia and to trigger macromolecular leakage in vivo. Interestingly, HBP regulation of barrier may not be selective for PMN, and in fact, endothelial cells themselves are now a reported source of HPB (61). It is therefore possible that endothelia may self-regulate permeability through HBP under some conditions, and that mediators found within the inflammatory milieu may also increase endothelial permeability. Similarly, PMNs were observed to significantly alter endothelial permeability by release of glutamate, following FMLP activation. This crosstalk pathway appears to be of particular importance for the regulation of the vascular barrier of the brain (“blood brain barrier”). In fact, treatment of human

brain endothelia with glutamate or selective, mGluR group I or III agonists resulted in a time-dependent loss of phosphorylated vasodilator-stimulated phosphoprotein (VASP) and significantly increased endothelial permeability. Glutamate-induced decreases in brain endothelial barrier function and phosphorylated VASP were significantly attenuated by pretreatment of human brain endothelia with selective mGluR antagonists. Even in an in vivo hypoxic mouse model, the pretreatment with mGluR antagonists significantly decreased fluorescein isothiocyanate-dextran flux across the blood-brain barrier, suggesting that activated human PMNs release glutamate and that endothelial expression of group I or III mGluRs function to decrease human brain endothelial VASP phosphorylation and barrier function (62).

A recently described gene regulatory pathway revealed a critical role for BMK1/ERK5 in maintaining the endothelial barrier and blood vessel integrity: A targeted deletion of big mitogen-activated protein kinase1 gene (BMK1) (also known as ERK5, member of the MAPK family), in adult mice leads to disruption of the vascular barrier. Histological analysis of these mice reveals that, after BMK1 ablation, hemorrhages occurred in multiple organs in which endothelial cells lining the blood vessels became round, irregularly aligned, and, eventually, apoptotic. In vitro removal of BMK1 protein also led to the death of endothelial cells partially due to the deregulation of transcriptional factor MEF2C, which is a direct substrate of BMK1. Additionally, endothelial-specific BMK1-KO leads to cardiovascular defects identical to that of global BMK1-KO mutants. Taken together, these studies identify the BMK1 pathway as critical for endothelial function and for maintaining blood vessel integrity (63).

### 3.1.4. Effect of adenosine receptor activation on endothelial barrier function

In vitro studies of endothelial permeability suggested, that activation of a specific endothelial adenosine receptor, the $AdoRA_{2B}$, leads to a barrier resealing response following PMN transmigration (64). Thus studies were able to show that of the four different adenosine receptors that are expressed by endothelia, only the $AdoRA_{2B}$ is selectively induced by hypoxia (35). Activation of the $AdoRA_{2B}$ is associated with increases in intracellular cAMP concentration due to the activation of the adenylate cyclase (23). By inhibition of cAMP formation, the resealing of the endothelial barrier during PMN transmigration can be obviated (64). Such increases in cAMP following activation of the $AdoRA_{2B}$ lead to an activation of protein kinase A (PKA) (8). Further studies revealed a central role of PKA-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a protein responsible for controlling the geometry of actin-filaments (65). Adenosine-receptor mediated phosphorylation of VASP is responsible for changes in the geometry and distribution of junctional proteins, thereby affecting the characteristics of the junctional complex and promoting increases in barrier function (8, 66).

### 3.1.5. Vascular barrier during inflammation

Ongoing inflammatory responses are characterized by dramatic shifts in tissue metabolism. These changes include large fluctuations in energy supply and demand and diminished availability of oxygen (28). Such shifts in tissue metabolism result, at least in part, from profound recruitment of inflammatory cell types, particularly myeloid cells such as PMN and monocytes. The majority

of inflammatory cells are recruited to, as opposed to being resident at, inflammatory lesions, and myeloid cell migration to sites of inflammation are highly dependent on hypoxia-adaptive pathways (28, 67). Consequently, much recent attention has focused on understanding how metabolic changes (e.g. hypoxia) relate to the establishment and propagation of the inflammatory response.

Many parallels exist between hypoxic and inflamed tissues (68). For example, during episodes of hypoxia, PMN are mobilized from the intravascular space to the interstitium, and such responses may contribute significantly to tissue damage during consequent reperfusion injury (62, 69, 70). Moreover, emigration of PMN through the endo- and epithelial barrier may lead to a disruption of such tissue barriers (71-73) and such a setting creates the potential for extravascular fluid leakage and subsequent edema formation (74, 75). In contrast, transcriptional pathways mediated by hypoxia-inducible factor (HIF) may serve as a barrier-protective element during inflammatory hypoxia. For example, experimental studies of murine inflammatory bowel diseases have revealed extensive mucosal hypoxia and concomitant HIF-1 activation during colitis (76). Mice engineered to express decreased intestinal epithelial HIF-1 exhibit more severe clinical symptoms of colitis, while increased HIF levels were protective in these parameters. Furthermore, colons with constitutive activation of HIF displayed increased expression levels of HIF–regulated barrier-protective genes (multidrug resistance gene-1, intestinal trefoil factor, CD73), resulting in attenuated loss of barrier during colitis in vivo. Such studies identify HIF as a critical factor for barrier protection during mucosal inflammation and hypoxia (76).

## 3.2. Connexin

### 3.2.1. Connexins, connexons and gap junctions

Among the various cell contact mediating protein complexes like tight junctions, desmosomes and cell adhesion molecules, gap junctions form conduits between adjacent cells and allow direct intercellular communication without transit through the extracellular space. Gap junctions are composed of connexin protein subunits. Six connexin subunits can form a hemichannel (connexon) in the plasma membrane that can dock to another hemichannel in the plasma membrane of an adjacent cell, in order to assemble a complete gap junctional channel.

The connexin (Cx) gene family comprises 20 members in the mouse and 21 members in the human genome (Table 1). The structure of connexin genes is relatively simple. An untranlslated exon 1 is separated by an intron of different length from exon 2, containing the uninterrupted coding region and the 3´-untranslated region (3´-UTR). In some untranslated regions and the reading frame are spliced. The connexin subunit is a four-transmembrane spanning protein, harboring two extracellular, one cytoplasmic loop and one cytoplasmatic N- as well as C-terminal region. Within both extracellular loops, the order of three cystein residues is highly conserved (except for Cx31): first loop: (C-$X_6$-C-$X_3$-C) and second loop (C-$X_5$-C-$X_5$-C). Opposing cysteines in both loops were suggested to form disulfide-bridges stabilizing the loops during the docking of two connexons).

Two different nomenclatures are in use: Cx are designated according to the species from which they were derived and their theorectical molecular mass (in kDa) (77). On the other hand, connexins can be divided into subgroups (α, β or

γ) with respect to their extent of sequence identity and length of the cytoplasmic loop (78). Connexins are then abbreviated with Gj for gap junction and numbered according to the order of discovery.

The first step in gap junction formation involves the assembly of newyl synthesized connexins into connexons in either the endoplasmatic reticulum or trans-Golgi network. The connexons then travel in vesicular structures along microtubules to the plasma membrane, where they insert. This insertion process appears to occur randomly all over the plasma membrane (79). The final step in gap junction formation involves the lateral diffusion of connexons in plasma membrane to the outer margins of the gap junction plaque, where they can dock to form complete gap junction channels (80).

Various compounds, generally less than 1000 Da, can be exchanged by passive diffusion through gap junctional conduits, i.e. metabolites, ions, second messengers (e.g. $Ca^{2+}$, $IP_3$, cAMP, ATP) water and electrical impulses (81-84). The ionic conductance allows rapid intercellular spread of action potentials in excitable cells, such as heart cells and neurons. They also bidirectional signaling between oocytes and granulosa cells to coordinate maturation of the ovarian follicle (85), and the maintenance of osmotic balance in lens fibers (86, 87).

Formation and degradation of gap junctions is a very dynamic process with reports of half-lives of less than 2h in cultured cells and tissues (88-92). Therefore, the regulation of gap junction assembly and turnover is likely to be critical in the control of intercellular communication.

### 3.2.2. Innexins and pannexins

Innexins form another family of gap junctional proteins that are only expressed in invertebrates (D. melanogaster or C. elegans) and do not show any sequence similarity to connexins (93). In total, 25 Caenorhabditis elegans (Ce-Inx and 8 Drosophila melanogaster (Dm-Inx) innexins have been identified. Mutant analysis and molecular characterization have shown that innexins are not functionally equivalent and are engaged in similar roles to connexins, e.g. in synaptic transmission, embryonic and postembryonic developement (94).

Pannexins (Panx) are orthologs of the invertebrate innexins. Vertebrates express both connexins and pannexins. Three pannexins are known, pannexin 1,2 and 3. Despite the lack of significant sequence homology, strong similarities exist at the structural and functional level with canonical gap-junction proteins. Thus pannexins share the same membrane topology with innexins and connexins as well as the hallmark of regularly spaced cysteine residues in the two extracellular loops connecting the transmembrane domains. Whereas the connexins contain three such residues, pannexins contain only two, thus resembling in this respect innexins, although the spacing of the cysteine residues in the second extracellular loop of pannexins diverges from that of innexins (94). Northern blot analysis indicates that Panx1 and Panx2 transcripts are detected in many rodent tissues, including brain and spinal cord (where Panx2 is particularly abundant), eye, thyroid, prostate, and kidney. The widespread distribution of Panx1 has been confirmed by probing human tissues with the highest levels being found in heart, gonads, and skeletal muscle. These results are at variance with those found in rodents, in that no signal was detected in skeletal musle and heart by Northern blot. Panx3 presents the most

restricted pattern of distribution and has been detected only in skin, which is devoid of Panx1 and Panx2 mRNA (94).

Expression of pannexins in paired oocytes revealed that at least one of them, Panx1, forms functional intercellular channels. In addition, Panx1, exhibits hemichannel acitivity in single oocytes, i.e. a membrane channel is formed by allowing flux of ions and larger molecules, like ATP, across the cell membrane (95). Studies revealed that pannexins are mechanosensitive and higly permeable to ATP, to an extent that ATP carries current (96). Support for an involvement of pannexin 1 in ATP release comes from a unique model system, the erythrocytes. ATP release from erythrocytes is part of a physiological control loop regulating peripheral vascular perfusion. When exposed to low oxygen content erythrocytes release ATP (97). The study of mechanisms of ATP release in erythrocytes has several advantages. Human erythrocytes do not contain vesicles, ruling out a vesicular ATP release mechanisms. Erythrocytes do not contain Cx43, however they express other possible ATP channels including CFTR and P2X7 (98). In addition, erythrocytes express pannexin 1 (98). A channel activity could be recorded form membrane patches excised from erythrocytes, which is consistent with pannexin 1 channels, in assumption that pannexin 1 exerts a role in eryhrocytes as a pannexon (99). It was shown that ATP release from erythrocytes can be attenuated by carbenoxolone, a gap junction blocker (99), but it has to be considered, that carbenoxolone is not specific and does not allow discrimination between channels. These aspects are very interesting, but should be examined more exactly in the future.

### 3.2.3. Extrajunctional connexons

Severeal studies have detected connexons at the cell surface that are not part of an intercellular gap junction (100-103). In general, although connexons are probably on the surfaces of most cells they remain primarily in a closed state. Curiously, connexon activation might be a common response to metabolic inhibition (104-106). Evidence for active channels that are composed of extrajunctional connexons was first obtained by the in vitro expression of cloned connexins. Some examples: Expression of connexon 46 in Xenopus laevis oocytes resulted in membrane depolarisation, which was followed by eventual cell death (100). The oocytes also became permeable to Lucifer yellow. These observations indicate the presence of open extrajunctional connexons. Cx46 containing connexons were also depolarized by decreases in extracellular $Ca^{2+}$, and so it seems that connexon activity is modulated in a manner that is similar other ion channels. Studies of retinal neurons also provided evidence for open extra-junctional connexons. Similar to the oocytes that expressed Cx46, cultured teleost horizontal cells developed large voltage-sensitive plasma-membrane conductances became permeable to Lucifer yellow when extracellular $Ca^{2+}$ was lowered. Subsequently, connexons that function as transmembrane channels have been observed in Novikoff heptoma cells (107), astrocytes (108) and ventricular myocytes (109).

Further member of the connexin family have also been shown to induce conductance through single membranes in Xenopus oocytes and in mammalian cell-expression systems - Cx50 (110), Cx45 (111), skate Cx35 (112) and Xenopus oocytes Cx38 (113, 114). So the formation and activation of connexons seems to be a general property of connexins in various cell types.

### 3.2.4. “Connexin diseases”

Deficient or impoper gap junctions have recently been associated with a variety of diseases including some forms of neuropathy, hereditary deafness, cataracts, skin disease, heart disease and cancer (115).

For example, ablation of Cx26 or Cx30 protein in cochlea both leads to hearing loss (116, 117), which corresponds to nonsyndromic deafness in patients carrying mutations in their Cx26 and Cx30 coding regions. Defects in Cx26, Cx30.3 and Cx31 have been linked to skin disease erythrokeratoderma (118).

Mutation within Cx32 gene are associated with X-linked peripheral nerve disorder Charcot-Marie tooth syndrome, which results from demylination of Schwann cells that surround peripheral neurons (119). Cx32 deficient mice also can show neuropathy at older age and are 25-fold more susceptible to carcinogen-induced liver cancer (115). A further example exists for Cx46 and Cx50, which are expressed in lens fiber cells of human and mouse eyes. Null mutation in the mouse (120), as well as single base mutations in the human orthologues both lead to connexin specific cataract formations (121, 122).

### 3.2.5. The “cardiovascular connexins”

In the vascular wall, four connexins (Cx37, Cx40, Cx43 and Cx45) have been detected in a pattern that varies depending on the cell type and the compartement (123-133). The vascular wall consists of two communication departments, the smooth mucle and the endothelium, both of which are connected with gap junctions. The endothelium consists of continuous monolayer of cells, lining the luminal surface of the entire vascular system, and which provides a structural and metabolic barrier between the blood and the

underlying tissues. In large arteries, Cx40 and Cx37 are widely distributed within the vascular wall whereas Cx43 shows a more heterogenous expression pattern (123, 129). Cx37 and Cx40 are the only connexins expressed by the mouse aortic endothelium and, recently the contribution of these proteins has been studied in transgenic mice lacking Cx40 (Cx40-/-) and/or Cx37 (Cx37-/-) in the aortic endothelium (132, 134). The intercellular dyes was found to be altered in Cx40-deficient aortic endothelium, under conditions leading to upregulation of Cx37 (130). Other dye transfer experiments, testing interendothelial cell communication in aortic segment of wild-type mice showed that both Cx37 and Cx40 are crucial for endothelial cell communication. The two connexins are mutually dependent on each other for optimal expression in vascular endothelium, inasmuch as elimination of either Cx40 or Cx37 resulted in a decrease of the non-ablated connexin, whereas both Cx37 and Cx43 are increased in the media layer of Cx40(-/-) (134).

HeLa cells, stably transfected with mouse Cx37, Cx40, Cx43 and Cx45 coding DNAs were demonstrated to be permeable to either the negatively charged Lucifer yellow (443 kDa; 2-) and the positively charged neurobiotin (287 kDa, 1+). Furthermore, is was corroborated that Cx40 hemichannels were not able to couple with Cx43 hemichannels in a heterotypic manner (135). Interestingly, Cx45 channels exhibit unusual high voltage sensitivity after application of various transjunctional voltages (136). This might reflect to the dual expression and function of Cx45 on the one hand in cardiomyocytes/endothelial cells of the cardiovascular system, on the other hand, in neurons of the central nervous system (137, 138).

Gap junction proteins may also coordinate the mechanical contractions of smooth muscle cells, possibly to insure a proper modulation of the vasomotor

tone of the vessel wall. Vasoconstriction and vasodilatation travel rapidly along the vessel network, due to the conduction of signals between endothelial and/or smooth musle cells through gap junctions. Vascular smooth muscle cells of both resistance and conduit arteries express predominantly Cx43 and Cx45, albeit Cx37 and Cx40 have also been reported in some vessels (125, 131).

The four “cardiovascular” connexins demonstrate profound heterogeneity in phenotypic correlations between man and mouse. One explanation for this discrepancy might be the fact that after genomic deletion two of the four connexin-deficient mice (Cx37-/-, Cx40-/-, Cx43-/- and Cx43-/-) die in utero (Cx45 (137, 139)) or shortly after birth (Cx43 (140)). Thus is was not possible to study the function of adult mouse hearts after loss of Cx43 and C45.

Only Cx37(-/-) and Cx40(-/-) animals were viable and fertile except for the Cx37(-/-) females, which suffer from female infertility (85).

To investigate the function of Cx43 in endothelial cells, an endothelial-specific Cx43 deletions was studied in mice. In one study, loss of Cx43 in the endothelium was found to cause hypertension and bradycardia (141). This hypotension was associated with a marked elevation in the plasma levels of nitrate, plasma angiotensin I and angiotensin II. In another study, lack of Cx43 in endothelium appeared to have no effect on resting blood pressure (142).

Loss of Cx45 blocks the vascular development after normal inititation of vessel formation, and impairs the differentiation of smooth muscle cells (137). Cx45-/- mice also showed a narrowing or the absence of the dorsal aorta and rare blood vessels in the yolk sac, due to reduced levels of hematopoiesis, possibly caused by defective TGFβ signalling (137).

In mice lacking Cx40 (143, 144), loss of this protein was shown to impair the conduction of dilatory signals along arterioles, indicating that Cx40 plays a role

in the propagation of the vasodilations. The Cx40-deficient mice were also hypertensive, indicating also a role of Cx40 in the control of blood pressure. Moreover, a spontaneous and irregular vasomotion was observed in a few Cx40-deficient mice that lead to arteriolar constriction, thus contributing to increase vascular resistance (145). Cx40(-/-)-deficient mice showed a tendency toward arrhythmias (143, 144), whereas the Cx37(-/-)-deficient mice were unaffected with regard to heart function (85). Interesingly, the Cx37(-/-)/Cx40(-/-) double deficient mice also die around birth due to vascular abnormalities with pronounced dilatation of blood vessels (134), since both connexins are coexpressed in endothelial cells.

In the meantime, other studies have been started to investigate whether downregualtion of either Cx43 or Cx45 in the hearts of Cx40-deficient animals aggravated the already existing arrhythmias. Therefore Cx40(-/-) mice were crossed with mice heterozygous for Cx43 or Cx45. These Cx40(-/-)/Cx43(+/-) mice (146) and Cx40(-/-)/Cx45(+/-) mice both showed abnormal cardiac conduction and morphogenesis leading to neonatal death in most of these animals (1). Thus, the 50% reduction of Cx43 or Cx43 expression (in heterozygous mice) crossed into Cx40(-/-) background aggravated the Cx40(-/-) phenotype strongly. This dose-dependent effect is not obvious in the corresponding Cx40(+/+)/Cx43(+/-) and Cx40(+/+)/Cx45(+/-) controls (1, 146).

The generation of mice lacking either Cx37 or Cx40 (134, 143, 144, 147) or Cx45 (132) has provided evidence for the biological function of gap junctions in the development and/functional maintenance of mouse vasculature.

In the kidney the juxtaglomerular region accomodated smooth muscle cells, endothelial cells, mesangial cells, macula densa cells and the renin-producing cells that are located in the distal part of the afferent arterioles. All these cell

types are connected by gap junctions which also connect the endothelial and the renin-producing cells of the afferent arteriole (148-151). mRNAs for nine connexins species, referred to as Cx26, Cx32, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45 and Cx46, have been found in the kidney (148, 152). For example, with regard to vessels, Cx43 is expressed by the endothelial cells of medium size and small kidney arterioles, but not by the media cells of latter vessels, barely, by glomerular capillaries (153).

### 3.2.6. Phosphorylation of connexins

Many connexins (Cx31, Cx32, Cx37, Cx40, Cx43, Cx45, Cx50 and Cx56) have been shown to be phosphoproteins by either a shift in their elektrophorectic mobility or direct incorporation of $^{32}P$ (154, 155). Functional data related to phosphorylation has been reported for Cx32, Cx43, Cx45 and Cx56. The C-terminal region of the connexin proteins appears to be the primary region that becomes phosphorylated. However, Cx56 can be phosphorylated within the cytoplasmatic loop region, in addition to its C-terminal domain (156). No reports of phosphorylation of the N-terminal region of connexin have been presented (157). Cx26 is the only connexin that has been reported to not be phosphorylated (158) which may be due to the fact that it is the shortest connexin and only a few C-terminal tail amino acids after the fourth transmembrane domain that could interact with cytoplasmic signalling elements.

Several reports have been shown that Cx43 has a half-life in the range of 1-3h in cultured cells or in tissues (89, 90, 92). Some investigators have shown that Cx43 is differentially phosphorylated throughout its life cycle in homeostatic cells (159). Under normal conditions, Cx43 is phosphorylated on multiple

serines. There have been a number of experimental observations that support the hypothesis that hemichannel gating can be modulated by phosphorylation. In reconstituted lipid vesicles, it has been shown that dephosphorylation of Cx43 leads to the opening of Cx43 hemichannels. The C-terminal region of Cx43 appears to be the primary region that becomes phosphorylated.

PKC activators (e.g. TPA) increase Cx43 phosphorylation and decrease gap junction communication in a number of different cells (91, 160-162). TPA treatment has been shown to reduce permeability of Cx43 channels via the phosphorylation of serine 368 (S368) (163) and dramatically decreases gap junction assembly (91).

PKA has been shown to phosphorylate Cx43 at S368 and S372 (163-165) in vitro. In Novikoff cell, TPA-activated PKC prevents dye uptake through Cx43 hemichannels (107). In solitary horizontal cell from catfish retina, application of dopamine, which activates cAMP-dependent protein kinase, suppresses hemichannel currents (103). Fibroblast growth factor-2, which decreases cardiomyocyte gap junctional permeability and increases Cx43 phosphorylation, increased the colocalization of PKCε with Cx43 (166). Phosphorylation of Cx43 by MAP kinase resulted in channel closure (167). Casein kinase 1, particularly the δ isoform, has been shown to interact with phosphorylated Cx43 on serine(s) 325, 328, or 330 (168) in vitro. Cx43 may also be a direct substrate for casein kinase 1 in vivo, as these rediues are major phosphorylation sites of cellular Cx43 (168).

Nitric oxide acting through a guanylate cyclase also closes hemichannels (80).

Certainly, other kinases phosphorylate Cx43. There is experimental evidence that Cx43 can be phosphorylated on at least 12 of the 21 serines (157) and 2 of the tyrosines (when the src kinase is active (169)) in the cytoplasmic tail region

(amino acids 250-382). It is possible that multiple of kinase may phosphorylate the same residue. For example, serine 255 can be phosphorylated by MAP and cdc2 (170, 171).

Thus, considerable evidence indicates that Cx43 is a highl phosphorylated and higly regulated protein.

### 3.2.7. Connexin communication in lymphocytes and between lymphocytes and endothelial cells

Peripheral T, B, and NK lymphocytes purified from healty human express connexin 43. Lymphocytes derived from secondary organs express, in addition, relatively low levels of Cx40 (172). Flow cytometry approaches using anti-peptide connexin antibodies that bind to the extracellular loops confirmed that the connexins are located on the cell surface (172). These observations are also highly suggestive of the presence of unapposed connexon hemichannels on lymphocytes with independent alternative communication functions such as the ATP-mediated propagation of calcium waves (173, 174). Calcein, a gap junction permeant fluorescent dye, was observed to transfer between contacting lymphocytes; importantly this direct transfer was inhibited by two categories of gap junction inhibitors (172) and thus implying direct communication across these junctions. Exposure of lymphocytes for 10-50 h to connexin mimetic peptides, specific inhibitors for Cx43, markedly reduced the production of immunglobulins (Ig) G, M and A in mixed cultures of human T and B lymphocytes (175). A small degree of intercellular channel rectification was observed in heterotypic and homotypic T and B cell cultures suggesting a role for lymphocyte gap junctions in the polarisation of the immune response (175). Interaction between lymphocytes and endothelial cells is a central feature of

vascular physiology. Blood-borne lymphocytes leave the circulation via specialised postcapillary vessels, the high endothelial venules (176). The active process of adhesion and transmigration across the endothelial cell layer are complex and involve a series of time dependent molecular mechanism which guarantee not only adhesion but also direct intercellular communication. Gap junctions are present in varying degrees of complexity along the vascular wall (177). Lymphocytes establish functional gap junction channels with endothelial cell in vitro during active transendothelial migration in a time dependent manner (178).

## 3.3. Nucleotides

### 3.3.1. Nucleotide and inosine signaling

Nuleotides are organic moleculs, which function as basic constituents of nucleid acids (DNA and RNA), but also take over essential regulatory functions in cells. A nucleotide consists of a pentose sugar (desoxyribose or ribose), a base (a purine or a pyrimidine) and a phosphate or a phosphate group. Of the nucleotides the nucleosides have to be distinguished, that only contain a base and a pentose, without a phosphate. A nucleoside can be derived by phosphohydrolysis of a nucleotide.

Purine signaling is presently a field of intense investigation. Adenosine as an extracellular nucleoside interacts via P1 receptors. Five P1 receptor subtypes have been identified until now, namely $AdoRA_1$, $AdoRA_{2A}$, $AdoRA_{2B}$ and $AdoRA_3$ and the growth hormone secretagogue receptor GHSR (179, 180). Extracellular nucleotides as signaling molecules interact through to different categories of P2-types of cell surface receptors: P2Y type G protein coupled

receptors, and P2X type ligand gated ion channels. Eight subtypes of P2Y receptors have been characterized at present, which are either activated by the adenine nucleotides ATP or ADP or respectively by the uridine nucleotides UTP or UDP. One P2Y subtype is activated by both, adenine and uridine nucleotides (181-184). However, there can be large differences between different species (e.g. between human and rat) with regard to P2 receptor selectivity for adenine or uridine nucleotides (185). Despite of the fact that the vascular endothelium and multiple cell types of the immune system (including PMN) express different P2 receptors (181, 186), only very little is known about the effect of nucleotide signaling on neutrophil-endothelial interaction. In fact, the cell physiology of these receptors and the precise function of the multiple P2 receptor subtypes remains to be understood (187). As currently selective blockers of these receptors are being synthesized, we can soon hope for a more precise understanding of how nucleotide signaling modulates immune function (188).

There is no unequivocal evidence that AMP can function as a signaling molecule. Inbe et al. suggested that the orphan receptor GPR80/99 may function as an AMP receptor (189). In contrast, there is now convincing evidence that GPR80/99 binds α-ketoglutarat, thereby functioning as receptor for citric acid cycle intermediates (190). In addition, other studies demonstrated that GPR80/99 does not bind adenosine or AMP (191). Therefore, there is now consensus that GPR80/99 is not a nucleotide receptor (192).

In contrast to adenosine, which has for long been considered an important modulator of immune function and inflammation, its major degradation product inosine was originally thought to have no biological effects. However, some recent studies have suggested that inosine may also affect immune function and tissue protection (193). Similarly to adenosine, inosine concentrations are

increased during hypoxia and during ischemia/reperfusion (193). As such, inosine was found to bind to the adenosine $AdoRA_3$ receptor subtype (P1 receptors) and cause mast cell degranulation (194) or cutaneous vasodilatation (195). In addition, inosine was found to be involved in the down-regulation of tissue damage under different inflammatory conditions in vivo via activation of $AdoRA_{2A}$ and $AdoRA_3$ adenosine receptors (196). However, specific inosine receptors have not yet been described. In addition, it remains unclear whether endogenously produced inosine is sufficient to exert tissue protective effects as the $AdoRA_3$ receptors only show a low affinity for inosine. However, interstitial concentrations of inosine may be high enough to activate $AdoRA_3$ receptors during pathophysiological states such as tissue hypoxia (194). In addition, it is possible that inosine signaling may contribute to the effects of adenosine signaling, since exogenous administration of large doses of inosine were found to decrease ischemia reperfusion injury in several tissues (193, 194, 197). However, the contribution of inosine generation and inosine signaling to the regulation of the vascular barrier function is currently unknown.

### 3.3.2. Nucleotide phosphohydrolysis is increased during hypoxia

The major pathway for extracellular hydrolysis of ATP and ADP is the ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) (198), previously identified as ecto-ATPase, ecto-ATPDase or CD39 (199, 200). It is now appreciated that CD39 and CD39-like molecules (NTPDases) are widely distributed in most tissues and have various orientations within the cell. For example, the catalytic site of NTPDases faces the extracellular milieu (NTPDase1-3) and/or the lumen of intracellular organelles such as the Golgi apparatus and endoplasmic reticulum (NTPDase4-7). NTPDase5 and 6 may

also be found on the plasma membrane and secreted following proteolytic cleavage. In addition to modulating extracellular nucleoside generation, CD39 has been considered in the modulation of platelet purinoreceptor activity by the sequential hydrolysis of extracellular ATP or ADP to AMP (200, 201). This thromboregulatory potential of CD39 has been demonstrated by the generation of mutant mice with disruption of the CD39 gene (45), and by a series of experiments where high levels ATPDase expression are attained by adenoviral vectors in the injured vasculature (202-204).

Ecto-5'-nucleotidase (CD73) is a membrane bound glycoprotein which functions to convert the adenine nucleotide AMP into adenosine, which in turn can activate transmembrane adenosine receptors or can be internalized through dipyridamole-sensitive carriers (23). These pathways have been shown to result in such diverse endpoints as regulation of endothelial barrier (64), stimulation of epithelial electrogenic chloride secretion (responsible for mucosal hydration) (205), and promotion of lymphocyte-endothelial adhesion (206). Targeted over expression of lymphocyte CD73 has recently revealed that increased adenosine resulting from enhanced CD73 expression could contribute to thymocyte apoptosis in adenosine deaminase deficiency (207). Endothelial cells of many origins express constitutive CD73. The primary function attributed to endothelial CD73 has been catabolism of extracellular nucleotides, although CD73 may also mediate lymphocyte binding under some circumstances (206).

As outlined above, multiple cell types release adenine nucleotides under conditions of limited oxygen availability. Extracellular nucleotides are metabolized to adenosine by CD39 and CD73 and subsequent activation of surface adenosine receptors has been shown to regulate endothelial and

epithelial barrier function (64). Microarray RNA analysis revealed an increase in CD73 and CD39 in hypoxic endothelial and epithelial cells (35, 48). Metabolic studies of CD39/CD73 function revealed that hypoxia enhances CD39/CD73 function as much as 6-fold over normoxia. Examination of the CD73 gene promoter identified a binding site for hypoxia-inducible factor-1 (HIF-1) and inhibition of HIF-1alpha expression by antisense oligonucleotides resulted in significant inhibition of hypoxia-inducible CD73 expression. Studies using luciferase reporter constructs showed a significant increase in activity in cells subjected to hypoxia, which was lost in truncated constructs lacking the HIF-1 site. Mutagenesis of the HIF-1alpha binding site resulted in a nearly complete loss of hypoxia-inducibility. In vivo studies in a murine hypoxia model revealed that hypoxia-induced CD73 may serve to protect the epithelial (48) and endothelial (51) barrier, since the CD73 inhibitor alpha,beta-methylene ADP (APCP) promotes increased intestinal permeability and $cd73^{-/-}$ mice show increased vascular permeability during hypoxia exposure. These results identify a HIF-1-dependent regulatory pathway for CD73 and indicate the likelihood that CD39/CD73 protects the epithelial and vascular barrier during hypoxia (35, 48, 51).

### 3.3.3. Adenosine concentrations are increased by hypoxia

Extracellular adenosine has been widely implicated in adaptive responses to hypoxia. Thus, it is well documented that adenosine tissue and plasma levels are increased during hypoxia, however, mechanisms of this response have yet to be further elucidated (30, 208). For example, in human volunteers exposed to ambient hypoxia ($SpO_2$=80% over 20 min), plasma adenosine concentrations increased from 21 to 51 nM in the presence of dipyridamole, an

inhibitor of adenosine reuptake (209). Similarly, when measuring adenine nucleotide concentrations in neurally and vascularly isolated, perfused skeletal muscles of anesthetized dogs, normobaric hypoxia is associated with increases of adenosine in the venous blood, but not of AMP, ADP or ATP (210). A possible role for adenosine during hypoxia may include vasodilatory characteristics. For instance, it has been suggested that elevations in intracellular nitric oxide as a result of $AdoRA_1$ activation may be responsible for the vasodilatory properties of adenosine (211-213). Due to its vasodilator properties, increases in adenosine tissue concentrations during hypoxia may promote blood flow to hypoxic tissues, thereby providing an innate adaptive pathway to hypoxia. In addition, there is evidence that hypoxia-induced vascular leak is, at least in part, controlled by adenosine. In vitro and in vivo studies indicated that endogenously generated adenosine, through activation of PMN adenosine $AdoRA_{2A/2B}$ receptors, functions as an anti-adhesive signal for PMN binding to microvascular endothelia (27, 36, 214). Other studies revealed that adenosine derived via nucleotide phosphohydrolysis is critical in eliciting decreases in endothelial paracellular permeability (64) and that such responses are dramatically enhanced by hypoxia (35, 48). In addition, some studies have suggested that uptake of adenosine by equilibrative nucleoside transporters (ENTs) in cultured myocardial cells are decreased during hypoxia. This might resemble an additional cellular strategy to increase extracellular levels of adenosine during hypoxia (215). However, this has not yet been investigated within the endothelium or the vasculature, and the physiological relevance of this adaptive pathway currently remains unclear.

### 3.3.4. Adenosine signaling effects are increased following hypoxia exposure

Several studies had demonstrated a functional role for hypoxia-regulated CD39 and CD73 in promoting endothelial permeability (35, 36, 48), however, the role of signal transduction via endothelial adenosine receptors in the post-hypoxic vasculature remained unclear until recently. In order to identify changes in endpoint signaling (i.e. adenosine receptor activation) in the post-hypoxic endothelium, studies profiled the relative expression of adenosine receptors in normoxic and hypoxic endothelial cells. Interestingly, these experiments demonstrated that the $AdoRA_{2B}$ was selectively induced by hypoxia, and that all other isoforms were either not changed ($AdoRA_3$) or significantly down-regulated ($AdoRA_1$ and $AdoRA_{2A}$) (35). Such microarray results were verified by real-time PCR and measurement of surface protein levels and consistently confirmed selective induction of the $AdoRA_{2B}$ receptor by hypoxia. To determine whether such hypoxia-induced $AdoRA_{2B}$ expression was functional, endothelial permeability assays were employed using the selective $AdoRA_{2B}$ antagonist MRS1754 (216) and demonstrated a significantly shift in the adenosine dose-response curve suggesting that $AdoRA_{2B}$ responses are amplified in post-hypoxic endothelia. However, further studies in vivo to verify these in vitro findings in genetically engineered animals using targeted deletion of individual adenosine receptors have yet to be performed to follow up on these observations.

## 3.4. PMN

### 3.4.1. Neutrophils as a source of extracellular nucleotides

Historically, activated platelets have been thought to serve as the primary source of extracellular adenine nucleotides (ATP, ADP, AMP) during inflammation (37). Platelets have well-established mechanisms for activated degranulation of dense granule constituents (ATP, ADP) as a means to elevate extracellular nucleotides for a variety of physiologic and pathophysiologic functions. The first studies implicating inflammatory cells other than platelets as a source of extracellular nucleotides dates back to the early 1990's in work by Madara et.al., who identified a soluble factor from activated neutrophils (PMN, polymorphonuclear leukocytes) which elicited electrogenic chloride secretion (i.e. water transport) in cultured epithelial cells (217, 218). They termed the active factor in the soluble fraction NDS for "neutrophil-derived secretagogue". In subsequent studies, they were able to capture the activity, purify to homogeneity and scale the system for mass spectrometry. Based on structural, biophysical and functional evidence, NDS was identified as 5'-AMP (205).

These original studies demonstrating functional responses (e.g. electrogenic chloride secretion) to PMN-derived 5'-AMP were somewhat surprising, since no 5'-AMP receptor existed. Rigorous pharmacologic studies were employed to define potential signaling pathways through 5'-AMP. A breakthrough was made when Madara et. al. inhibited PMN-derived 5'-AMP activity by incubation with adenosine deaminase (205). These results implicated adenosine as the active factor in PMN-derived 5'-AMP. Subsequent studies revealed that inhibitors of the ecto-5'-nucleotidase (CD73), blocked activity found within PMN-derived 5'-AMP (205). These results suggested the necessity of CD73 as an intermediate

step in the formation of active adenosine. The adenosine liberated by this enzymatic process is then made available to activate any of four subtypes of G-protein coupled adenosine receptors (termed A1, A2A, A2B and A3). It was subsequently shown that the rate-limiting enzyme CD73 is more widely expressed than originally thought and may broadly contribute to a number of inflammatory conditions (219), particularly in the setting of hypoxia and stabilization of the transcription factor hypoxia-inducible factor (HIF, see later).

More recent studies revealed that the active fraction originally defined as PMN-derived 5'-AMP may actually be ATP. Indeed, studies directed at understanding vascular barrier function during conditions of inflammation or hypoxia identified the existence of a soluble fraction derived from activated PMN which amplified the resealing of vascular barrier function following subjection of cultured endothelial cells to periods of hypoxia. A screen of HPLC purified fractions identified this activity as PMN-derived ATP (35). Like 5'-AMP, this activity was functionally linked to CD73 and to adenosine receptors. This was puzzling, since CD73 was not known to utilize ATP as a substrate. Rather, these studies identified the ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) (198), previously identified as ecto-ATPase, ecto-ATPDase or CD39 (199, 200) as the central surface enzyme in this reaction. CD39 is expressed on the endothelium (35) and it's primary role had been to modulate platelet purinoreceptor activity by the sequential hydrolysis of extracellular ATP / ADP to AMP (200, 220). Studies in both cd73- and cd39-null mice have confirmed many of the original in vitro studies and strongly implicate this metabolic pathway in a variety of inflammatory conditions, particularly as they relate to inflammatory hypoxia (35, 51).

### 3.4.2. Mechanisms of PMN-derived ATP release

Studies directed at understanding mechanisms of ATP release by activated PMN considered several potential mechanisms, including exocytosis of ATP containing vesicles, transport via connexin hemichannels, transport through nucleoside transporters, or direct transport through ATP-binding cassette (ABC) proteins (221). Initially, it was determined that ATP does not localize with known granule markers in PMN. While isolated granules from resting PMNs contained greater than 95% of proteolytic enzyme activity markers, ATP levels within the isolated granules were nearly undetectable. Moreover, PMN fractions containing cytosolic markers contained ATP concentrations that were higher than 5 mM, thereby suggesting that activation-dependent ATP release likely occurs independent of classical PMN degranulation. Based on these findings, a pharmacologic approach was employed to examine potential mechanisms. Brefeldin A (BFA), a general vesicular secretion inhibitor did not influence activated PMN ATP secretion. Likewise, neither the nucleoside transport inhibitor dipyridamole nor the general ABC transport inhibitor verapamil significantly influenced PMN ATP secretion (222).
Based on previous reports suggesting that connexin hemichannels may serve as ATP release channels (173) and the observation that PMN express surface connexins (223), studies examined the non-specific gap junction inhibitor 18-α-glycyrrhetinic acid (18αGA), which revealed that 18αGA inhibited ATP release in a concentration-dependent manner (222). Likewise, connexin-mimetic peptides specifically directed against Cx43, but not Cx40 (173), significantly blocked ATP liberation from activated PMN (222). In addition to their role as gap-junction proteins, recent studies indicate that Cx43 connexons are also active in single plasma membranes and can function in intercellular signaling as

ATP release channels (173). Studies addressing Cx43 Ser-368 phosphorylation in intact PMN showed prominent phosphorylation in resting PMN and protein phosphatase 2A-dependent dephosphorylation within minutes of PMN activation.

As proof of principle, PMN were isolated from tamoxifen-inducible Cx43 conditionally deleted mice (224) and examined for ATP release. These studies revealed that ATP release correlated with the degree of Cx43 expression. In total, these studies provide strong evidence that ATP release occurs through a conformational opening of membrane Cx43 hemichannels in response to PMN activation (222).

### 3.4.3. Functional implications of extracellular ATP derived from PMN

Studies directed at understanding extracellular metabolism of nucleotides in cell and tissue responses now suggest that a number of different cells can release ATP in an active manner, particularly when oxygen levels are limited (225). Most studies have suggested that ATP liberated by activated PMN is “auto-hydrolyzed” to AMP through PMN surface CD39. Further metabolism of AMP to adenosine requires an additional cell type to contribute CD73 (or other phosphatase activity such as alkaline phosphatase) activity as a means to generate adenosine. As such, PMN CD39 may function as an immunomodulatory control point, requiring close special relationship to CD73-positive cells (such as endothelia, epithelia, or lymphocytes). Alternatively, other phosphatases (e.g. alkaline phosphatase) also have AMP hydrolyzing activity. A direct comparison of CD73 and alkaline phosphatase activity in the mucosa revealed that while both enzymes hydrolyze AMP, CD73 appears to

predominate at physiologically-relevant concentrations of AMP (226). Nonetheless, PMN express plasma membrane alkaline phosphatase (227), and as such, PMN as a single cell population have the capacity to generate adenosine from ATP. It is not currently known how relevant this latter pathway is to overall ATP metabolism (Figure 1).

Adenosine exerts paracrine and autocrine functions on most cell types. Pathophysiologic conditions of hypoxia / ischemia result in numerous adenine nucleotide metabolic changes, and adenosine has a demonstrated role in organ function under such conditions. While the source of interstitial adenosine in hypoxic tissue has been the basis of much debate, it is generally accepted that the dephosphorylation of AMP by CD73 represents the major pathway of adenosine formation during oxygen supply imbalances (228). Adenosine production in the ischemic myocardium, for example, is attributable to activity of CD73 (228), and both CD73 activity and adenosine metabolism have been demonstrated in cardiac pre-conditioning by brief periods of ischemia (25). Specifically, adenosine is made and liberated into the extracellular milieu through both transcriptional and non-transcriptional mechanisms (229). It is appreciated that the adenosine made during intermittent hypoxia (HPC) occurs through non-transcriptional metabolic changes [e.g. inhibition of adenosine kinase, release through nucleoside transporters (22, 229)]. By contrast, chronic (non-intermittent) hypoxia results in the coordinated transcriptional induction of surface enzymes which metabolize extracellular nucleotides (23, 219).

Increased CD73 activity in ischemic preconditioning has been attributed to a variety of acute activation pathways (228), and recent studies provide direct evidence that CD73 is transcriptionally regulated by hypoxia in a variety of cells in vitro (47, 230, 231). Once liberated in the extracellular space, adenosine is

either taken up into the cell (through dipyridamole-sensitive carriers) or interacts with cell surface adenosine receptors (24, 25). Endothelial cells of many origins express adenosine receptors constitutively, primarily of the A2A and A2B subtypes (232), wherein vascular endothelial cells are one of the most enriched cell populations of A2BR.

In addition to regulating endothelial and epithelial barrier function, a recent study reported a role for PMN-dependent ATP release in directed movement of PMN (233). These studies demonstrated that human PMN release ATP at the leading edge of migrating cells and amplify chemotactic signals for directed cell orientation by feedback through P2Y2 nucleotide receptors. These studies suggested that PMN rapidly hydrolyze released ATP to adenosine that then acts through A3-type adenosine receptors, which are recruited to the leading edge and thereby promote cell migration. As such, ATP release and autocrine feedback through P2Y2 and A3 receptors provide signal amplification, controlling gradient sensing and migration of neutrophils. Such studies highlight an additional role of extracellular nucleotide release in modulating inflammatory functions of innate immune responses.

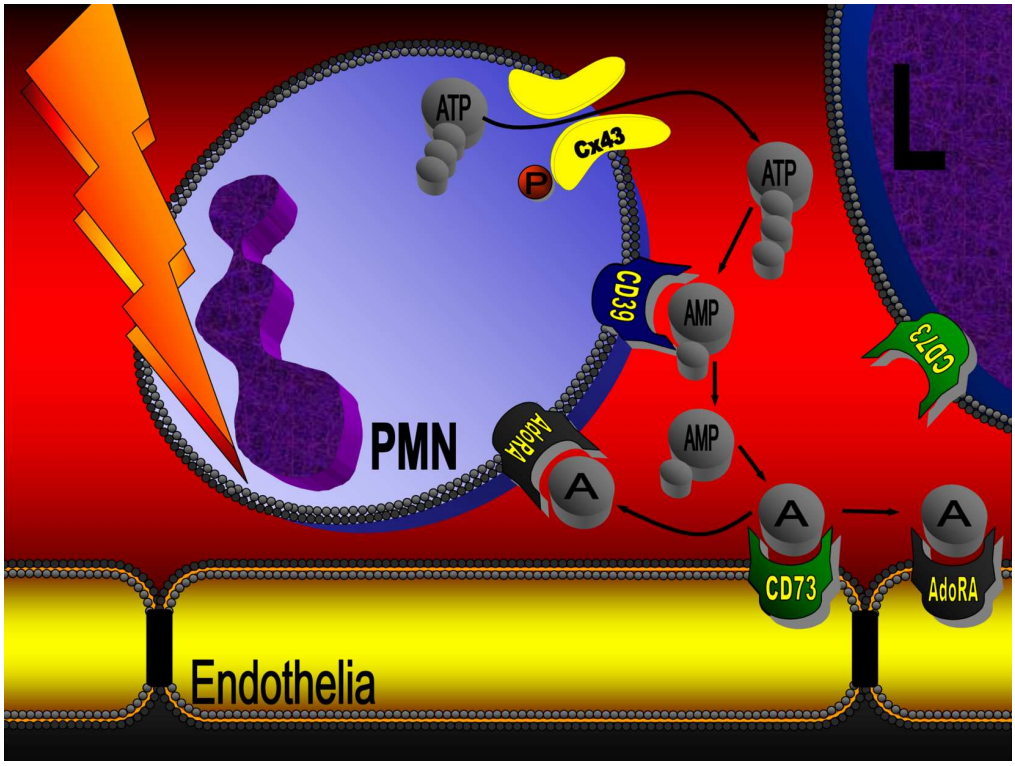



***Figure 1:*** *Proposed model of ATP release from PMN. Upon inflammatory activation, PMN release ATP through Cx43 hemichannels from the cytosol into the extracellular space. This process is controlled by phosphatase A2-dependent phosphorylation of connexin 43 hemichannels (Cx43, P). Extracellular ATP is then rapidly hydrolyzed to AMP by CD39 expressed on the surface of PMN. During neutrophil-endothelial interaction, further metabolism of AMP to adenosine requires ecto-nucleotidase activity (CD73), which for example can be contributed by endothelia or other myeloid cells (e.g. lymphocytes, L). As the final step in this cross-talk pathway, adenosine generated in this fashion can activate adenosine receptors on endothelia or PMN, resulting in a modulation of endothelial barrier function or neutrophil-endothelial adhesion during transmigration (222).*

## 3.5. ATP Release from Vascular Endothelia

Extracellular nucleotide and nucleoside levels are increased during conditions of limited oxygen availability (hypoxia) (26, 234-241). Particularly extracellular elevations of ATP are critical in vascular adaptation to hypoxia, as extracellular ATP can either signal directly to ATP receptors (242, 243) or is rapidly converted to adenosine leading to increased vascular nucleoside signaling (35, 36, 244-247). While previous studies have shown that during hypoxic conditions, adenosine stems mainly from ATP-phosphohydrolysis, (34, 35, 239, 240) cellular sources and mechanisms for extracellular ATP release during hypoxia are not well defined. As such, different cell types can contribute to elevations of vascular ATP during hypoxia, including circulating blood cells such as platelets, erythrocytes or inflammatory cells (e.g. PMN) (222). In addition, vascular endothelia may represent an important cellular source for extracellular ATP release during hypoxia (34) as they extend over a large surface area and are anatomically positioned at the interface between a hypoxic stimulus and the surrounding tissues (35).

At present, several studies have detailed different molecular mechanisms involved in cellular release of ATP into the extracellular milieu. Such mechanisms differ between multiple cell types (e.g. platelets, astrocytes, umbrella cells of the bladder) (186). For example, ATP release from umbrella cells of the bladder is dependent on mechanical pressure stimulation. This ATP release can be blocked by inhibitors of connexin hemichannels, ABC-protein family members, or nucleoside transporters (248). Other studies on cellular mechanisms of ATP release found that ATP release from neuronal astrocytes involves the gap-junction molecule connexin 43 (Cx43) (249). In addition to

their role as gap-junctional proteins, recent studies indicate that Cx43 connexons are also active in single plasma membranes and can function in intercellular signaling as ATP release channels (173). As such, genetic and pharmacological studies of neutrophil-dependent ATP release suggest a critical role of Cx43-hemi-channels in extracellular ATP release following inflammatory stimulation (222), which may play an important role in purinergic chemotaxis (233, 250).

In the present study, we tested the hypothesis that vascular endothelia represent an important source for extracellular ATP elevation during hypoxic conditions. To our surprise, we found that extracellular ATP release from vascular endothelia is attenuated following hypoxia exposure. Additional studies to address molecular mechanisms of endothelial-dependent ATP release suggested a functional contribution of Cx43 in endothelial ATP release. Moreover, hypoxia exposure of endothelia revealed that Cx43 expression is attenuated by hypoxia, while Cx43-Serine368 phosphorylation status is increased, which has previously been shown to change the Cx43 channels from an open to a closed status (163). These findings suggest that extracellular elevations of vascular ATP levels during hypoxia predominantly stem from other cell types than vascular endothelia (e.g. inflammatory cells, or red blood cells) (222, 233, 251, 252).

## 3.6. Hypoxia Inducible Factor

### 3.6.1 Control of extracellular nucleotide metabolism: critical role HIF

Adenosine is liberated in at high levels in the setting of hypoxia (219, 229). At the tissue and cellular level, hypoxia induces an array of genes pivotal to survival in low oxygen states. As a global regulator of oxygen homeostasis, the αβ heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1) facilitates both oxygen delivery and adaptation to oxygen deprivation (253).

HIF-1 is a member of the Per-ARNT-Sim (PAS) family of basic helix-loop-helix (bHLH) transcription factors. HIF-1 activation is dependent upon stabilization of an $O_2$-dependent degradation domain of the α subunit and subsequent nuclear translocation to form a functional complex with HIF-1β and cofactors such as CBP and its ortholog p300 (254). Under conditions of adequate oxygen supply, iron- and oxygen- dependent hydroxylation of two prolines (Pro564 and Pro 402) within the oxygen-dependent degradation domain (ODD) of HIF-1α initiates the association with the von Hippel-Lindau protein (pVHL) and rapid degradation via ubiquitin-E3 ligase proteasomal targeting (253). A second hypoxic switch operates in the carboxy terminal transactivation domain of HIF-1α where hypoxia blocks the hydroxylation of asparagine-803 and facilitates the recruitment of CBP/p300 (255).

For a number of years, it remained poorly understood how hypoxia might stabilize the expression of HIF. In the past several years, the molecular mechanisms of HIF activation have become clarified. These studies have defined three HIF-selective iron- and oxygen-dependent hydroxylation enzymes (PHD1-3) (on HIF prolines 564 and 402) within the oxygen-dependent degradation domain (ODD) of the HIF-α subunit (256, 257). The three enzymes

have different tissue distributions and, at least under conditions of over-expression, have distinct patterns of sub-cellular localization (258). PHD1 mRNA is expressed in many tissues, with especially high expression in the testes. Likewise, PHD2 mRNA is widely expressed, with particularly abundant expression in adipose tissue (258, 259). PHD3 mRNA is also expressed in many tissues, but is most abundant in the heart and placenta (258, 259).

When levels of $O_2$ fall below a critical threshold (hypoxia), the lack of PHD substrate ($O_2$) results in the accumulation of HIFα, which then associates with HIFβ. The HIF heterodimer translocates to the nucleus where it is made available to activate HIF-bearing gene promoters. Genes induced by HIF-1 include those necessary for cell, tissue and whole animal adaptive responses to hypoxia (253). These proteins include enzymes involved in anaerobic metabolism, the angiogenic cytokine vascular endothelial growth factor (VEGF), and inducible nitric oxide synthase (253).

The discovery of HIF-selective PHD's as central regulators of HIF expression has now provided the basis for potential development of PHD-based molecular tools and therapies (260). Pharmacological inactivation of the PHDs by 2-OG analogues is sufficient to stabilize HIF-α (260), but this action is nonspecific with respect to individual PHD isoforms. In vitro studies do suggest significant differences in substrate specificity. For example, PHD3 does not hydroxylate proline 564 on HIF-α, and comparison of enzyme activity in vitro showed that the ODD sequence is hydroxylated most efficiently by PHD2 (258, 259). These observations have generated an interest in identifying enzyme-modifying therapeutics. Indeed, a number of PHD inhibitors have been described, including direct inhibitors of the prolyl-hydroxylase (261), analogs of naturally occurring cyclic hydroxymates (262), as well as antagonists of α-keto-glutarate

(260). Based on extensive, mucosal diseases are excellent targets for PHD-based therapeutics (263). In particular, most evidence indicates that HIF activation results in an inflammatory-protective phenotype, including nucleotide metabolic pathways that promote tissue barrier function and stimulate the resolution of inflammation.

# 4. MATERIALS AND METHODS

## 4.1. Endothelial Cell Culture

Human microvascular endothelial cells (HMEC-1) and human umbilical vein endothelial cells (HUVECs) were cultured as described previously (32, 35).

## 4.2. Measurement of extracellular ATP release into the supernatant from HMEC-1

Confluent HMEC-1 monolayers were exposed to normoxia or indicated time periods of hypoxia (2% oxygen). These studies were carried out in the presence of the polyoxotungstate $Na_6[H_2W_{12}O_{40}]$ (POM-1, 100 µM), a highly potent non-specific inhibitor of ecto-nucleoside triphosphate diphosphohydrolases (239). The culture medium was removed, cells were washed twice and incubated as indicated in calcium containing HBSS, and samples from the supernatant were collected, shock frozen and stored at -80º C for further analysis. In subsets of experiments, intracellular ATP levels were measured. Here, confluent HMEC-1 monolayers were exposed to hypotonic lysis by addition of ice-cold water. Following centrifugation over 5 min at 13,000 rpm, the ATP content in the supernatants was quantified using a highly sensitive luciferase based technique (CHRONO-LUME, Chrono-Log Corp., USA, Haverton). Luciferase activity was measured on a luminometer (Turner Designs Inc., Sunnyvale, California, USA) and compared with ATP standards (35, 222).

### 4.3. Measurment of lactate dehydrogenase (LDH) in supernatants from HMEC-1

To assess lytic ATP release, the cytotoxicity detection kit (Roche Diagnostics, Germany, Mannheim) was used according to the manufacture's instructions (222). In short, confluent HMEC-1 monolayers were exposed to normoxic or hypoxic (2% oxygen) conditions over indicated time periods, then 100 µl of the substrate mixture from the kit were added to 100 µl of the collected protein-free supernatant. After an incubation period of 30 min the absorbance was measured at 490 nm. In control studies, normoxic HMEC-1 were treated with 2% Triton X-100 to assess maximal LDH release.

### 4.4. Pharmacological studies on mechanisms of endothelial-dependent ATP release

Confluent HMEC-1 were seeded on 6-well plates, culture medium was removed and cells were washed with calcium free 5M HEPES HBSS. In subsets of experiments, HMEC-1 monolayers were treated with 18-alpha-glycyrrhetinic acid (18αGA; Sigma Aldrich, 20µmol), Anandamide (Sigma Aldrich, 40µmol) or the phosphokinase C inhibitor bisindolylmaleimide (BIM, Sigma Aldrich, 10 µM). Furthermore, the non specific ABC receptor antagonist Verapamil (Tocris Cookson, 10µmol) and the non specific inhibitor of adenosine transporters dipyridamole (Sigma Aldrich, 1 µmol) were used. In other studies, the effects of connexin mimetic peptides for connexin 43 (SRPTEKTIFII, Biosource, Germany, Solingen, 50µmol) and connexin 40 (SRPTEKNVFIV, Biosource, Germany, Solingen, 50µmol) were used. After 20 min of incubation time at room temperature, ATP content within the supernatants was measured as described above.

### 4.5. Transcriptional analysis

To assess the influence of hypoxia on enthothelial connexin transcript levels, HMEC-1 monolayers were exposed to hypoxia (2% oxygen) over indicated time periods, followed by isolation of RNA and quantification of transcript levels by real-time RT-PCR (iCycler; Bio-Rad Laboratories Inc.). RNA was isolated by using Total RNA purification NucleoSpin RNA II according to the manufacturer's instructions (Marcherey-Nagel, Germany, Düren). RNA concentration was measured followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The PCR reactions contained 10 pM sense and 10 pM antisense oligonucleotides with iQ SYBR Green Supermix (Bio-Rad Laboratories). The target sequence was amplified using increasing numbers of cycles of 95℃ for 15s, 56℃ for 30s, 72 ℃ for 15s as described previously (35, 264). Realtime RT-PCR conditions and primer sequences are summarized in Table 2.

### 4.6. Western blot analysis

Cx43 expression and phosphorylation status were assessed by Western blot analysis using connexin 43 rabbit polyclonal antibody (Cell Signaling) or phospho-connexin 43 rabbit polyclonal antibody detecting serine 368 phosphorylated Cx43 (Cell Signaling) as described previously (222). In subsets of experiments, HMEC-1 were pre-treated with the phosphokinase C (PKC) inhibitor bisindolymaleimid during hypoxia or normoxia exposure (10μM) (265).

### 4.7. Immunoprecipitation

Surface Cx43 expression was assessed as described previously (222). In short, confluent HMEC-1 were exposed to normoxia or indicated time periods of hypoxia (2% oxygen). Monolayers were washed, surface proteins labeled with biotin, lysed and centrifugated to remove cell debris. Protein concentration was measured and immunoprecipitation was performed with 2 µg monoclonal rabbit anti-mouse connexin 43 antibody (Acris) and incubated overnight. Subsequently, 50 µl Protein G Microbeads (Miltenyi Biotec) were added and incubated for 30 minutes on ice. Magnetically separated immune complexes were eluated with 1x SDS gel loading buffer, transferred to nitrocellulose, and blocked overnight in washing buffer with 3% BSA and 2 µg/ml streptavidine. Membranes were enhanced by alkaline phosphatase.

### 4.8. Data analysis

Data were compared by two-factor ANOVA, or by Student's *t* test where appropriate. Values are expressed as the mean ± SD from at least three separate experiments.

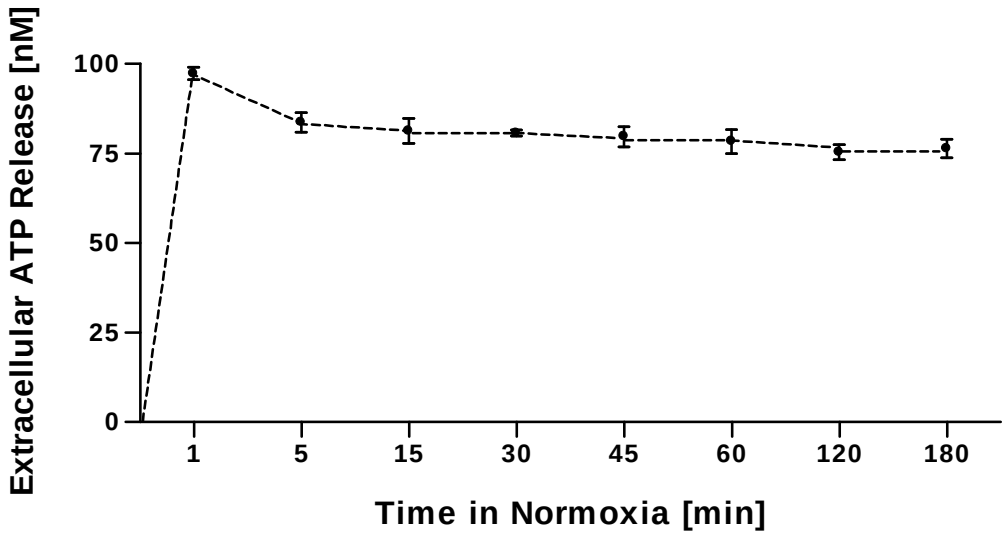
# 5. RESULTS

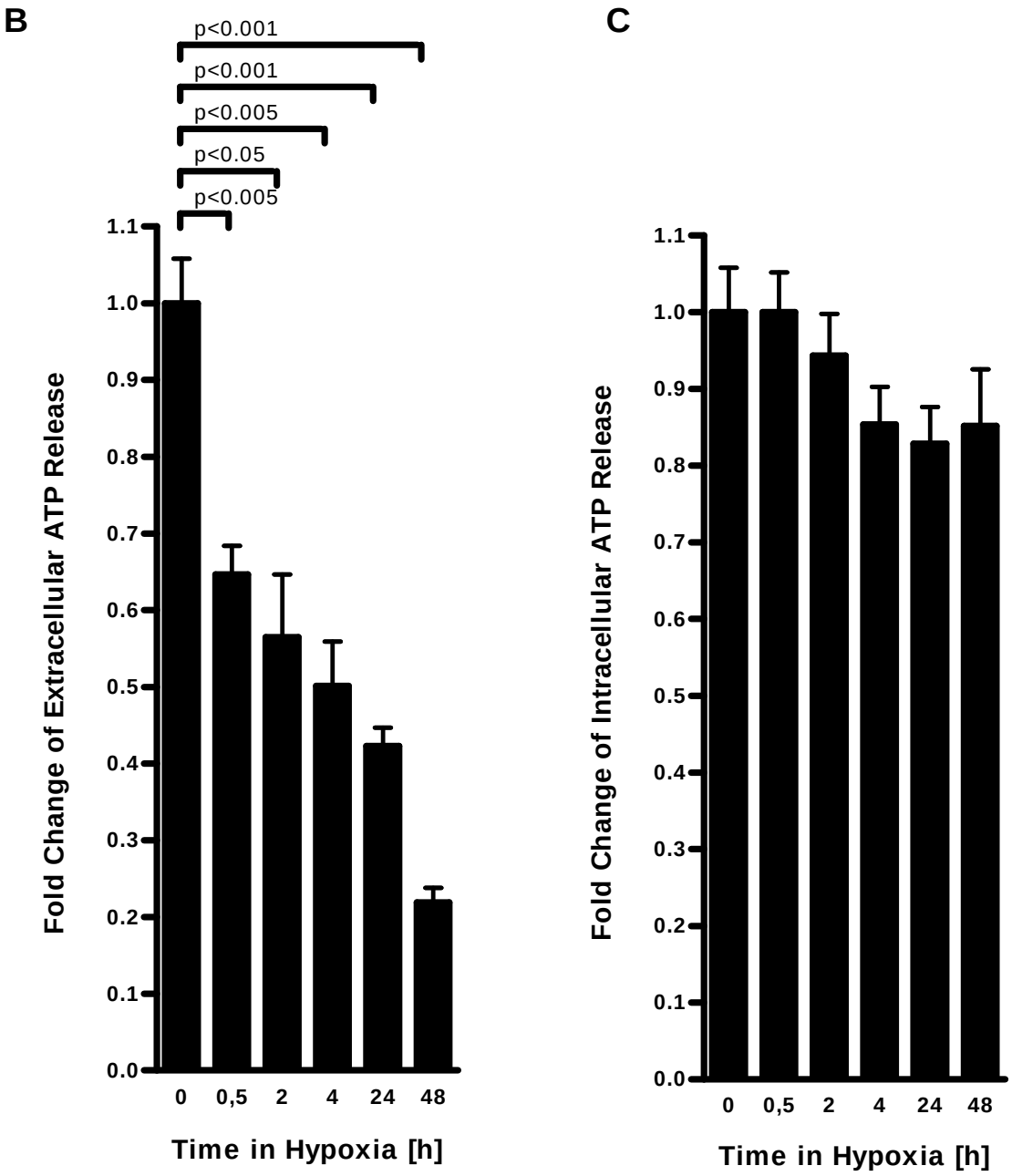
## 5.1. Extracellular ATP release from vascular endothelia is attenuated by hypoxia

To study the role of vascular endothelia in extracellular ATP release, we first measured the extracellular ATP content within the supernatant of vascular endothelia. For this purpose, the culture media from confluent HMEC-1 monolayers was replaced with calcium containing HBSS, and samples from the supernatant were collected at indicated time points, in the presence of POM-1, a highly potent ENTPDase inhibitor (239). As shown in Figure 2A, ATP levels within the supernatant immediately rise to approximately 90 nM and remained stable over the examined time period of 180 min. Therefore, ATP content in the supernatant from endothelia was measured in all subsequent experiments 30 min after the exchange of cell culture media by calcium containing HBSS. To study the influence of hypoxia on vascular ATP release, vascular endothelia were pre-exposed over indicated time periods to ambient hypoxia (2% oxygen), the media was replaced and ATP content was measured within the supernatants. Surprisingly, we found that extracellular ATP levels were lower in post-hypoxic endothelia in a time-dose dependent fashion, suggesting that endothelial ATP release is attenuated by hypoxia (Figure 2B). As next step, we lysed cells following different time-periods of hypoxia exposure and measured intracellular ATP levels. As shown in Figure 2C, intracellular ATP levels were unchanged in post-hypoxic endothelia, suggesting that the observed changes in ATP content within the supernatants were attributable to alterations of transcellular ATP gradients (e.g. loss of intracellular ATP levels resulting in a lower trans-cellular gradient). To study the contribution of lytic ATP release, we

measured LDH content in the supernatants of post-hypoxic endothelia. As shown in Figure 2D, LDH content in the supernatant did not change significantly with hypoxia exposure, suggesting that lytic ATP release did not account for differences between normoxic or post-hypoxic endothelial ATP release. Taken together, these studies demonstrate that ambient hypoxia of vascular endothelia results in decreased extracellular ATP release.

**A** **Figure 2**




B
p<0.001
p<0.001
p<0.005
p<0.05
p<0.005
Fold Change of Extracellular ATP Release
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
0,5
2
4
24
48
Time in Hypoxia [h]
C
Fold Change of Intracellular ATP Release
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
0,5
2
4
24
48
Time in Hypoxia [h]

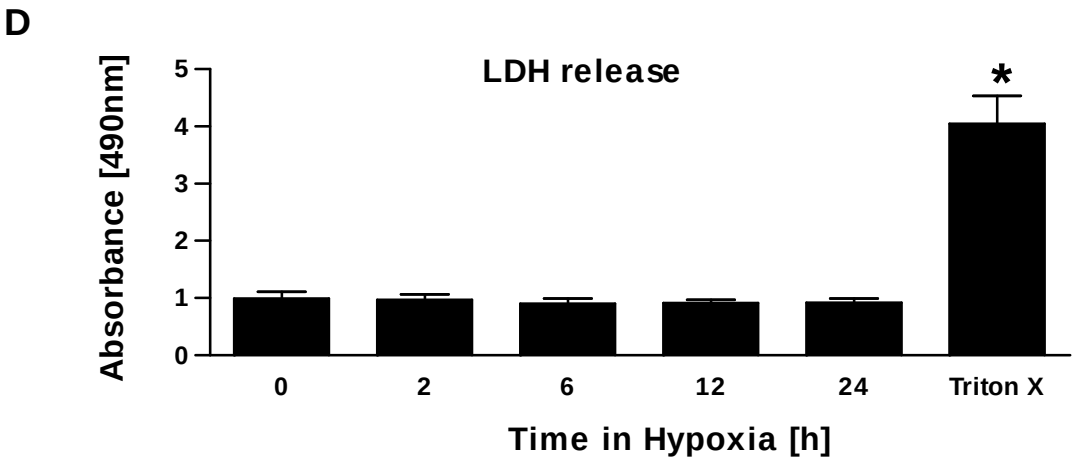

D
LDH release
Absorbance [490nm]
5
4
3
2
1
0
*
0
2
6
12
24
Triton X
Time in Hypoxia [h]

***Figure 2:*** *Endothelial ATP release during hypoxia****. A,*** *To study extracellular ATP release from normoxic endothelia, monolayers of confluent HMEC-1 were washed and the culture media was replace with calcium containing HBSS. ATP content from their supernatant was sampled at indicated time points and quantified using a luminometric ATP detection assay.* ***B,*** *To measure extracellular ATP release under hypoxic conditions, confluent HMEC-1 monolayers were exposed to hypoxia (2% oxygen) over indicated time periods. Culture media was replaced with calcium containing HBSS and the ATP content within the supernatant was measured after 30 min incubation time.* ***C,*** *For intracellular ATP measurement, confluent HMEC-1 monolayers were exposed to hypoxia over indicated time periods, culture medium was discarded and cells were lysed by adding ice-cold water. ATP concentrations were measured as above.* ***D,*** *Confluent HMEC-1 monolayers were exposed to hypoxia as indicated. To assess lytic ATP release, LDH concentrations within the supernatant were measured by an LDH detection kit. In control experiments, cells were lysed with Triton X-100 (*p<0.01, n=6 for all experiments).*

### 5.2. Mechanisms of endothelial ATP release

ATP exists in the cytoplasm at millimolar concentrations (221) and can be released extracellularly by several mechanisms, including transport via connexin hemichannels, through nucleoside transporters, or direct transport through ATP-binding cassette (ABC) proteins (222). As first step, we tested the effect of verapamil, an inhibitor of several ABC proteins and the multi drug resistance gene product (222). As shown in Figure 3A, we found no alteration in endothelial ATP release with verapamil treatment. Similarly, the nucleoside

transport inhibitor dipyridamole had no effect on endothelial ATP release (Figure 3B). Next, we measured ATP release of endothelia in the presence or absence of the non-specific gap junction inhibitor 18αGA (222). As shown in Figure 3C, addition of 18αGA resulted in a dramatic reduction of ATP release from normoxic endothelia. Similarly, treatment with the non-specific gap junction inhibitor anandamide (222) resulted in attenuated ATP release (Figure 3D). We extended these findings to define specific connexin contributions to endothelial ATP release. For these purposes, we used previously described connexin mimetic peptides specifically directed against Cx40 or Cx43 (222, 266). As shown in Figure 4A, the Cx40-specific connexin mimetic peptide did not alter ATP liberation from endothelia. By contrast, the peptides which block Cx43 showed a significant inhibition of ATP liberation (50.0±7% reduction, $p<0.01$ by ANOVA, Figure 4B). These results implicate Cx43 in ATP release from human endothelia.

**Figure 3**

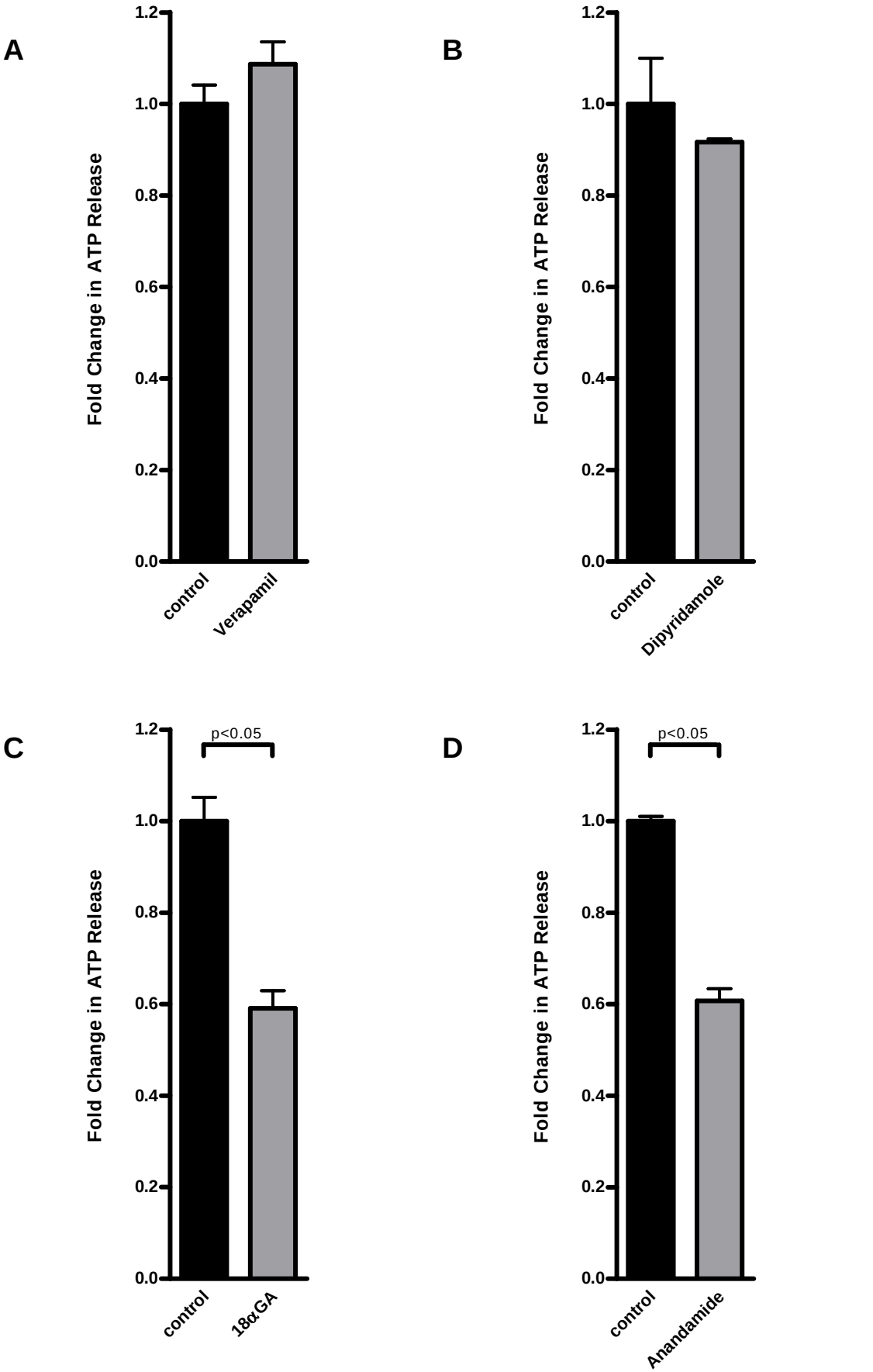

A
B
C
D
Fold Change in ATP Release
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
Verapamil
Dipyridamole
18αGA
Anandamide
p<0.05

***Figure 3:*** *Molecular mechanisms of endothelial-dependent ATP release.* ***A-D,*** *Confluent HMEC-1 monolayers were washed and exposed to Verapamil (10 µmol) or Dipyridamole (1 µmol),, 18-alpha-glycyrrhetinic acid (18αGA, 20 µmol) or Anandamide (40 µmol) over 20 min. ATP content within the supernatant was measured by a luminometric ATP detection assay and compared with untreated control cells (n=6).*

**Figure 4**

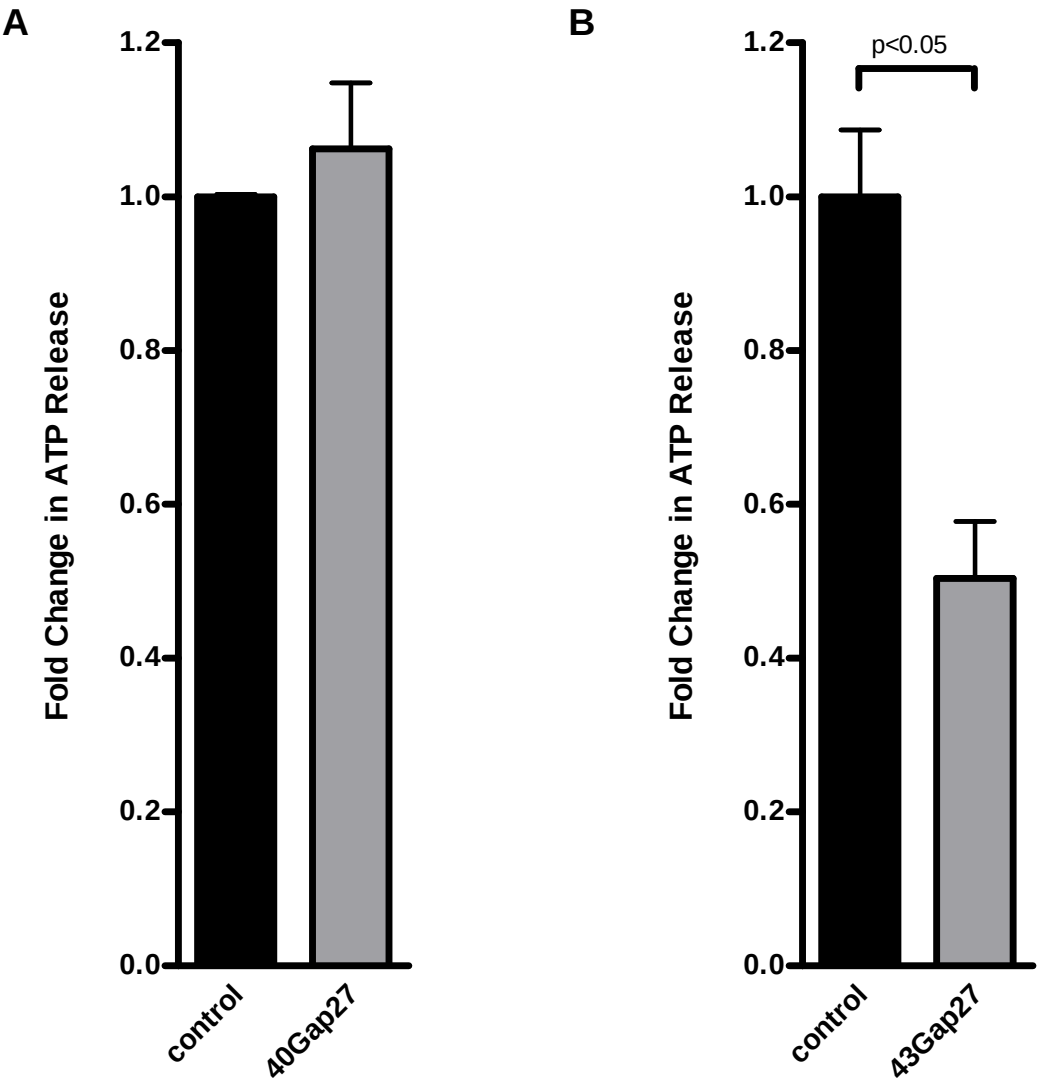


***Figure 4:*** *Connexin-mimetic peptides in endothelial ATP release.* ***A, B,*** *Confluent HMEC-1 monolayers were washed and treated with connexin-mimetic peptides (A: Cx40 peptide, SRPTEKNVFIV, 50 µmol; B: Cx43 peptide, SRPTEKTIFII, 50 µmol). ATP content within the supernatant was measured by a luminometric ATP detection assay after an incubation period of 20 min and compared with control HMEC-1 treated with 50 µM bovine albumin (n=6).*

### 5.3. Modulation of endothelial connexin expression by hypoxia

Based on our observations of attenuated ATP release by endothelia following hypoxia exposure and our data showing that gap-junctional inhibition is associated with attenuated endothelial ATP release, we next studied transcriptional consequences of hypoxia on endothelial connexin expression. Here, we first performed a transcriptional screen of known connexin molecules (1) using realtime RT-PCR to compare normoxic or hypoxic connexin expression of HMEC-1 (Table 2). Serendipitously, these studies revealed a selective repression of Cx43 transcript levels with hypoxia exposure (Figure 5, 2% oxygen, 12h of hypoxia), while transcript levels of other connexins were not observed. As shown in Figure 6A, these results could be confirmed with different time periods of hypoxia exposure and in a different human endothelial cell line (HUVECs, Figure 6B). Moreover, Western blot analysis showed time-dependent repression of Cx43 protein (Figure 6C) and immunoprecipitation localized attenuated Cx43 protein levels to the cell surface (Figure 6D). As previous studies had shown that PKC-dependent phosphorylation of the serine368 residue of Cx43 is associated with a functional change of Cx43 channels from the open to the closed state, (163, 267) we next studied Cx43-serine368 phosphorylation status by Western blot analysis using a serine368

phospho-specific antibody for Cx43. As shown in Figure 6E, hypoxia was also associated with increased phosphorylation of Cx43-Serine368. Consistent with previous reports, this response could be attenuated with the PKC inhibitor BIM (163, 267). Taken together, these studies reveal hypoxia-dependent repression of Cx43 transcript in conjunction with increased phosphorylation of Cx43-Serine368, suggesting that hypoxia attenuates endothelial ATP release by transcriptional repression and phosphorylation of Cx43.

**Figure 5**

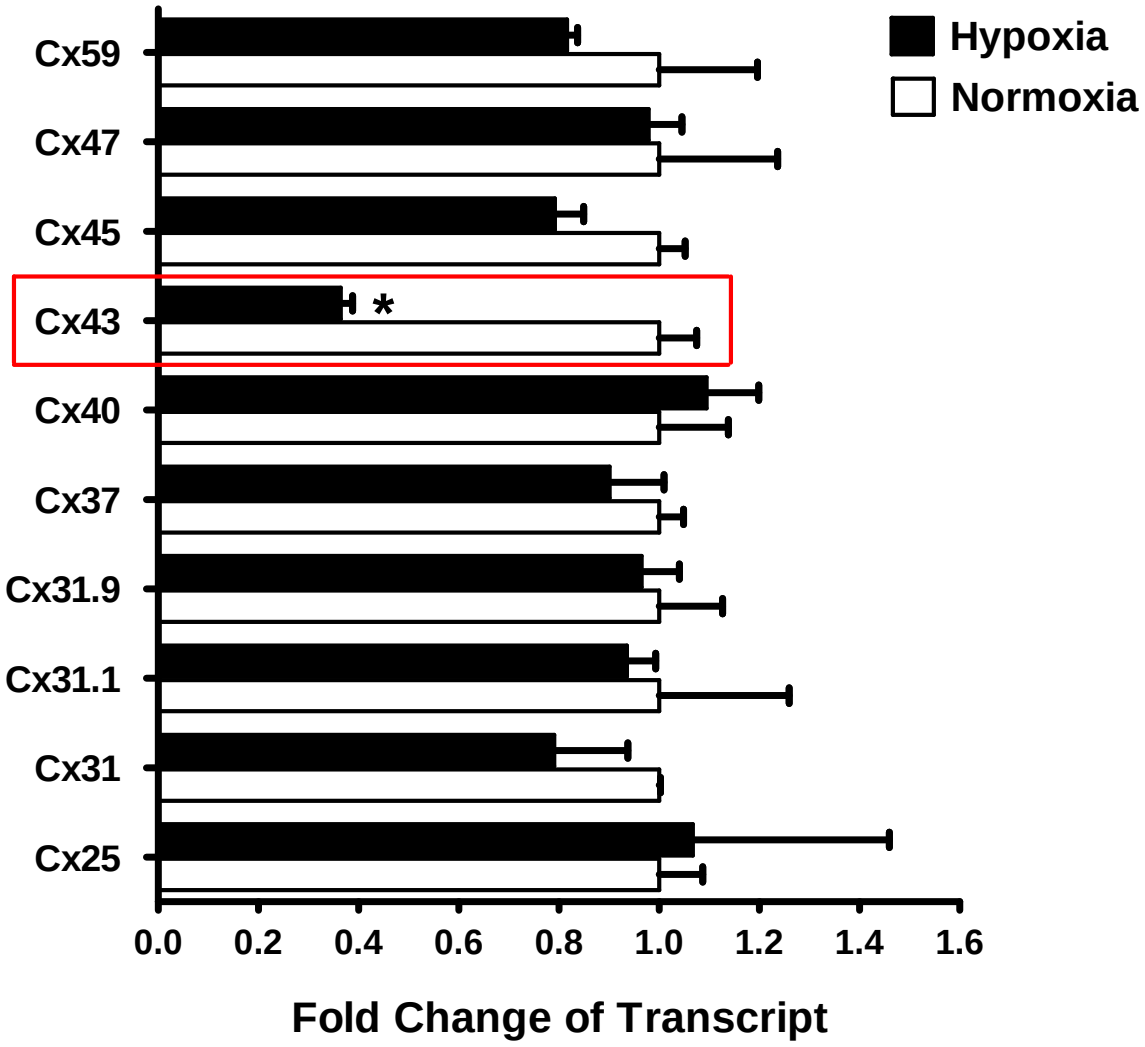
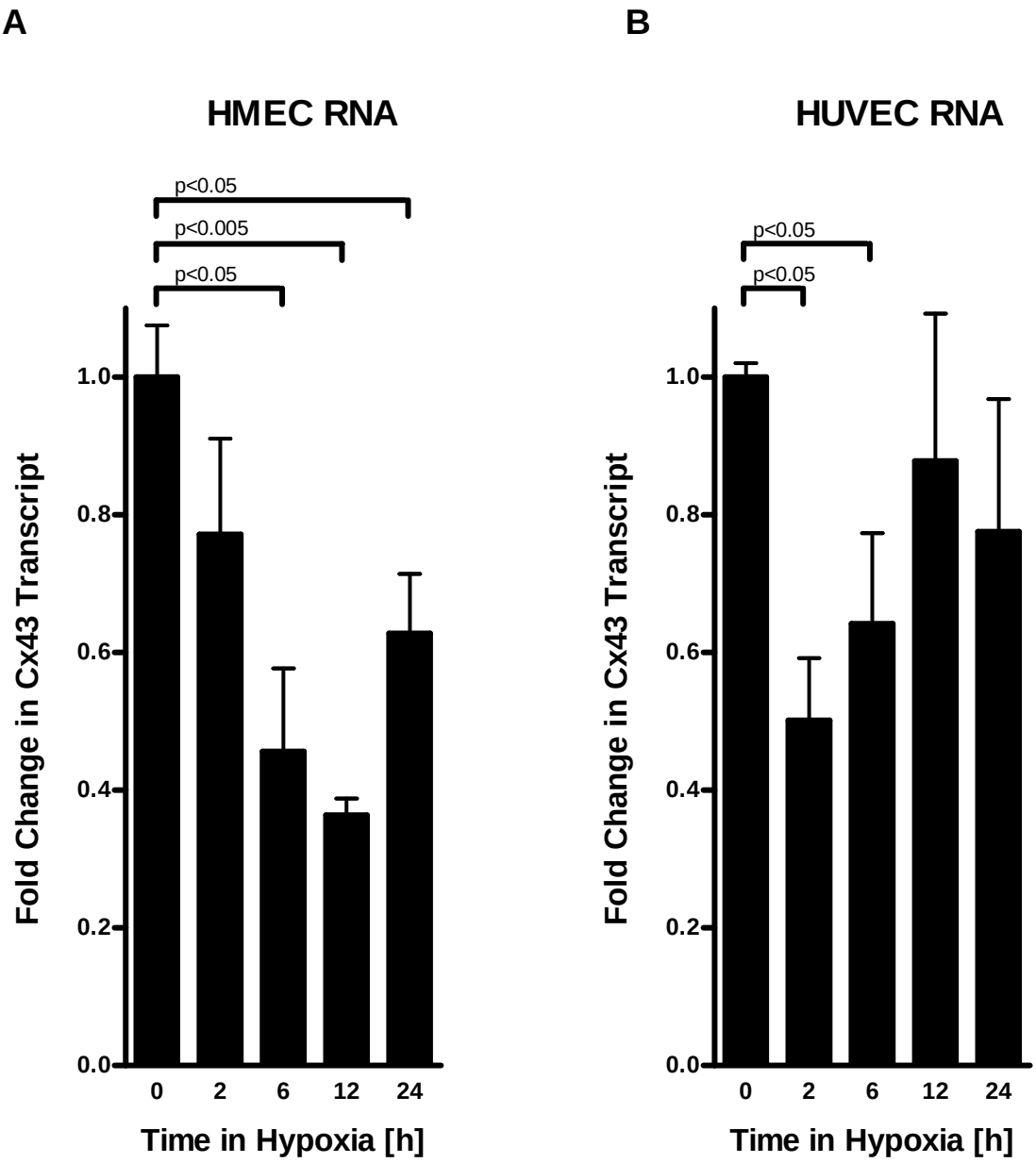



***Figure 5:*** *Endothelial connexin expression. Confluent HMEC-1 monolayers were exposed to normoxia or hypoxia (12h). Total RNA was isolated and real-time reverse-transcriptase polymerase chain reaction was employed to screen for transcriptional modulation of connexin expression. Data were calculated relative to an internal control gene (β-actin) and are expressed as fold change over normoxia at each time point. Results are derived from 3 experiments in each condition.*

**Figure 6**


A
HMEC RNA
p<0.05
p<0.005
p<0.05
Fold Change in Cx43 Transcript
1.0
0.8
0.6
0.4
0.2
0.0
0
2
6
12
24
Time in Hypoxia [h]
B
HUVEC RNA
p<0.05
p<0.05
Fold Change in Cx43 Transcript
1.0
0.8
0.6
0.4
0.2
0.0
0
2
6
12
24
Time in Hypoxia [h]

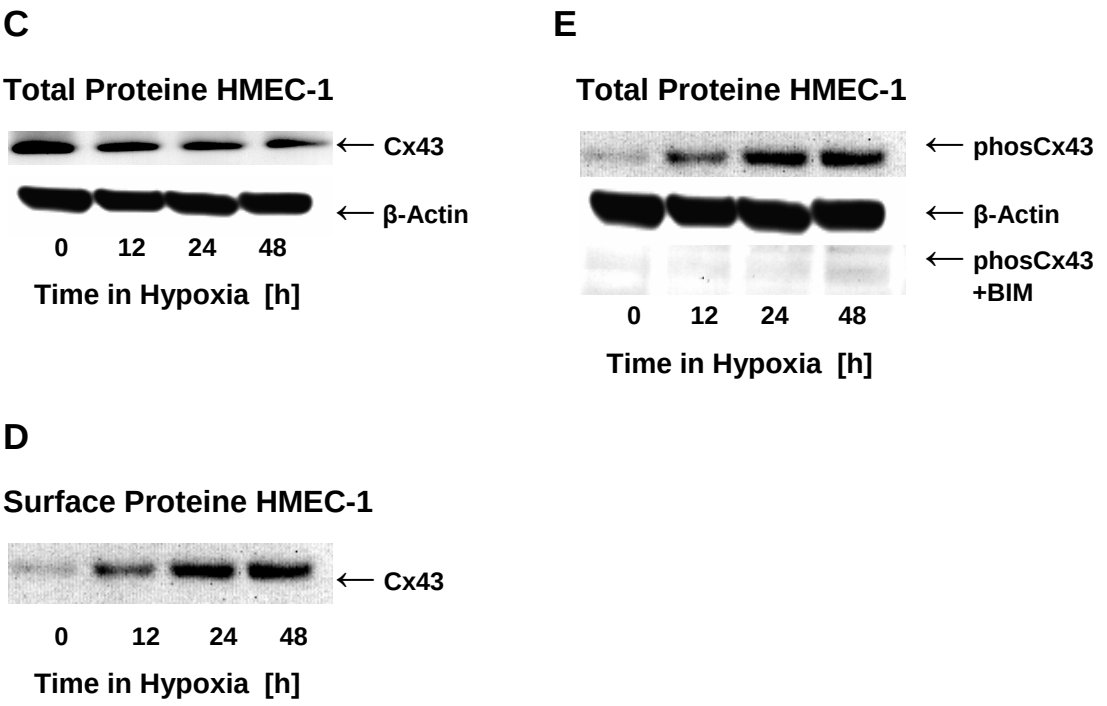



***Figure 6:*** *Influence of hypoxia on connexin 43 expression.* ***A, B,*** *Confluent HMEC-1 or HUVEC monolayers were exposed to normoxia or hypoxia (2% oxygen) over indicated time periods. Total RNA was isolated and transcriptional responses were assessed by real-time reverse-transcriptase polymerase chain reaction. Data were calculated relative to an internal housekeeping gene (β-actin) and are expressed as fold change over normoxia at each time point. Results are derived from 3 experiments in each condition.* ***C,*** *Confluent HMEC-1 monolayers were exposed to hypoxia over indicated time periods. Cells were lysed and proteins were resolved by SDS-PAGE and transferred to PVDF-membrane. Membranes were probed with a connexin 43 antibody, proteins were detected by chemiluminescene. The same blot was reprobed for β-actin as a control for protein loading. A representative experiment of 3 is shown.* ***D,*** *Confluent HMEC-1 monolayers were exposed to hypoxia over indicated time*

*periods. Monolayers were washed, surface proteins were biotinylated, and cells were lysed. Connexin 43 was immunoprecipitated, followed by addition of Protein G Microbeads. Proteins were resolved by SDS-PAGE and resultant Western blots were probed with Streptavidin. A representative experiment of 3 is shown.* ***E,*** *Confluent HMEC-1 monolayers were exposed to normoxia or hypoxia over indicated time periods. Cells were lysed and proteins were resolved by SDS-PAGE and transferred to PVDF-Membrane. Membranes were probed with phospho-connexin 43 antibody specific for phosphorylated ser368, and proteins were detected by chemiluminescene. The same blot was probed for β-actin as a control for protein loading. A representative of 3 is shown. In subsets of experiments, cells were pretreated with the protein kinase C inhibitor bisindolylmaleimide (10 µM; +BIM).*

### 5.4. Functional consequences of Cx43 phosphorylation on endothelial ATP release

We next pursued studies to address whether hypoxia-associated phosphorylation of Cx43 contributes to attenuated ATP release from vascular endothelia. Here, we measured endothelial ATP release in following PKC inhibition with BIM to attenuate Cx43-Serine368 (see above) under normoxic or hypoxic conditions (2% oxygen over 24h). These studies revealed that already under normoxic conditions ATP release was significantly increased following PKC inhibition (Figure 7) as compared to untreated normoxic HMEC-1 ($p<0.05$). While hypoxia exposure of untreated HMEC-1 was associated with a significant attenuation of ATP release ($p<0.001$), this response was completed abolished following PKC inhibition with BIM. In fact, BIM treated HMEC-1 had higher ATP levels within the supernatant than untreated normoxic HMEC-1

($p<0.05$). Taken together, these data suggest a functional contribution of Cx43 phosphorylation in attenuating endothelial ATP release during conditions of hypoxia.

**Figure 7**

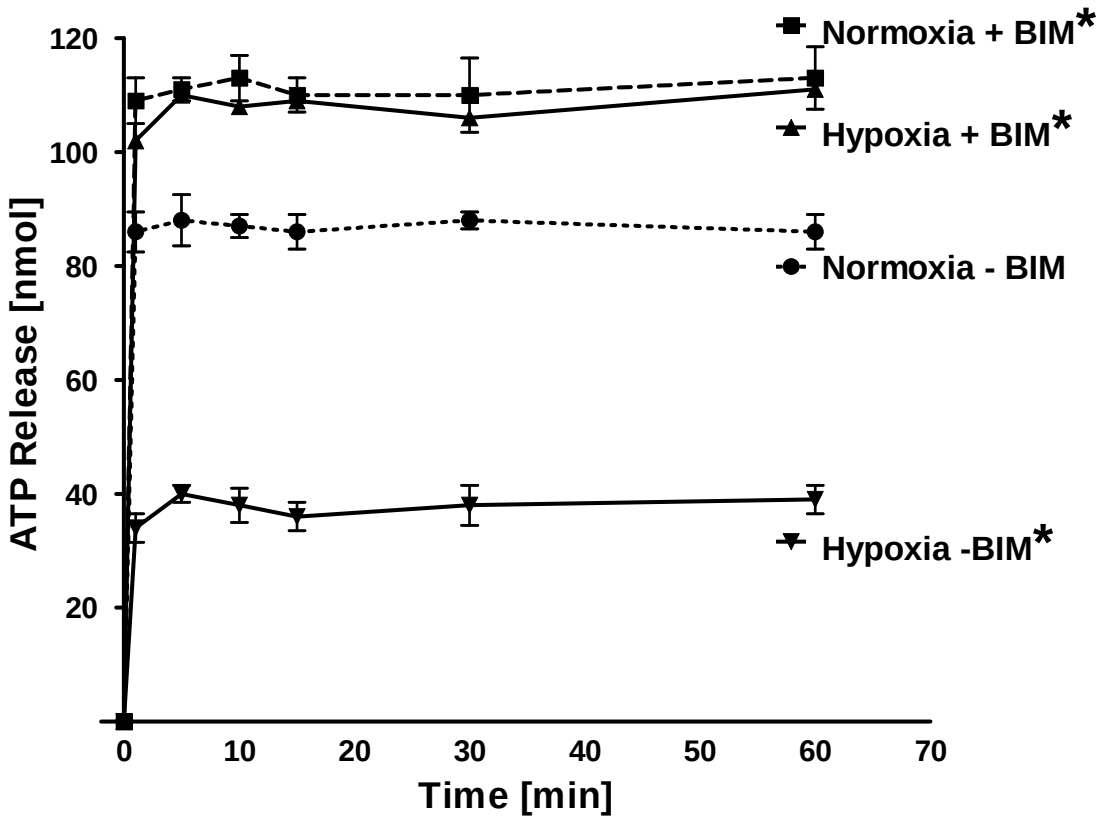



***Figure 7:*** *ATP release from HMEC treated with the protein kinase C inhibitor bisindolylmaleimide (BIM). To study the role of Cx43 ser398 phosphorylation status in ATP release from endothelia, monolayers of confluent HMEC-1 were treated with BIM (+BIM, 10 µM) or vehicle control (-BIM), exposed to normoxia or hypoxia (24h, 2% oxygen), washed and the culture media was replace with calcium containing HBSS. ATP content from the supernatant was sampled at*

*indicated time points and quantified using a luminometric ATP detection assay (*p<0.05 compared to Normoxia – BIM; n=6).*

**Table 1**

| chr | GJ | Cx |
|---|---|---|
| 6 | | hCx23 |
| 6 | | hCx25 |
| 13 | GJB2 | hCx26 |
| 7 | GJE1 | hCx30.2 (hCx31.3) |
| 13 | GJB6 | hCx30 |
| 17 | GJA11 | hCx31.9 |
| 1 | GJB4 | hCx30.3 |
| 1 | GJB3 | hCx31 |
| 1 | GJB5 | hCx31.1 |
| X | GJB1 | hCx32 |
| 15 | GJA9 | hCx36 |
| 1 | GJA4 | hCx37 |
| 10 | | hCx40.1 |
| 1 | GJA5 | hCx40 |
| 6 | GJA1 | hCx43 |
| 17 | GJA7 | hCx45 |
| 13 | GJA3 | hCx46 |
| 1 | GJA12 | hCx47 |
| 1 | GJA8 | hCx50 |
| 1 | GJA10 | hCx59 |
| 6 | | hCx62 |

Human Connexin (1)

## Table 2

| Human | Forward (5´-3´) | Reverse (5´-3´) | [bp] | [C°] |
|---|---|---|---|---|
| **ß-actin** | GGTGGCTTTTAGGATGGCAAG | ACTGGAACGGTGAAGGTGACAG | **162** | **56** |
| **Cx 25** | CAGGCCTCTTGCCGATTCAG | GTGGCCTCCACTTCCTATCA | **215** | **56** |
| **Cx 26** | AAGAAGTCGCTTGGGAATTT | GCTGAAGGGGTAAGCAAACA | **177** | **56** |
| **Cx 30** | TCCAGAAGGCAATACCAACC | CAATGCTCC TTTGTCAAGCA | **180** | **56** |
| **Cx 30.3** | AGAGGTGCATGGAGATCTTC | CAGCCTTCATTAGGACAGAG | **135** | **56** |
| **Cx 31** | CTTCCAGCAGCAGCAGGTCT | CACCAGCCTGAGCACAGTTG | **181** | **56** |
| **Cx 31.1** | ATCTACCTGGTGAGCAAGAG | GAGAGGAGGATGACTGTCTG | **162** | **56** |
| **Cx 31.3** | GAGAACCTTGCCTTGGTAGT | TTGTGTCTTCTGGTGCTCTC | **214** | **56** |
| **Cx 31.9** | GACCGTCTTCGTGCTCTTCT | AGCAGCTTCTGCGCCTCTTC | **153** | **56** |
| **Cx 32** | TCCGACAGCGTCTCCAATTA | TTGTGGCCAGCAAGCACTAT | **187** | **56** |
| **Cx 36** | GCATCAAGGAGGTGGAATGT | TTGAGTTCAGCCAGGTTGAG | **112** | **56** |
| **Cx 37** | CAACAGAGGGGTCCTGAGAA | CTGGAGAGGAAGCCGTAGTG | **182** | **56** |
| **Cx 40** | AGCAGGGGCAAGGAAATAGT | TACAGAGACCAGGCCAATCC | **162** | **56** |
| **Cx 40.1** | GCCGTCTTCAGCGTCTATGT | GAGGAGGAGGTGGATGATGT | **180** | **56** |
| **Cx 43** | AATTCAGACAAGGCCCACAG | CATGGCTTGATTCCCTGACT | **216** | **56** |
| **Cx 45** | ACGCTTGGATCTGGCAGTTC | TCAGTGAGCTGCTGCTTACC | **209** | **56** |
| **Cx 46** | TTCGAGCTGAAGCCGCTCTA | CGCCAGCATGAAGATGATGA | **111** | **56** |
| **Cx 47** | GACCACCGTGTGGATCTGAG | CGGCTAAGGAGAAGGCTGAG | **113** | **56** |
| **Cx 50** | CTCCACTCCATTGCTGTCTC | CGTAGGAAGGCAGTGTCTCT | **217** | **56** |
| **Cx 59** | AAGAGACCACAGCCTTAGGA | AGGAGTCCAGTCTAGAAGGA | **118** | **56** |
| **Cx 60** | CAGTGAAGGCAGCATGAGAG | TGACTGAAGGCAGAGGTGAG | **197** | **56** |

Human primer pairs used for real-time RT-PCR

# 6. DISCUSSION

Limited oxygen delivery to tissues (hypoxia) is common in a variety of disease states. A number of parallels exist between hypoxia and acute inflammation, including the observation that both influence vascular permeability.

The vascular endothelium is the predominant interface between the hypoxic insult and the surrounding tissue. Simultaneously, the endothelium provides the primary determinant of vascular permeability. Metabolic and transcriptional responses to inflammation are common denominators of multiple cardiovascular (268) and pulmonary diseases (269). In particular, adaptation to “inflammatory hypoxia” has become an area of intense investigation (235, 270, 271). Important in this regard, a consistent finding in hypoxic tissues increases extracellular nucleotide levels (54, 209, 210, 272). Previous studies have indicated that extracellular nucleotide metabolites may function as an endogenous protective mechanism during hypoxia and ischemia (21-23). One factor may be increased production of endogenous adenosine, a naturally occurring anti-inflammatory agent (23-25). Several lines of evidence support this assertion. First, adenosine receptors are widely expressed on target cell types (e.g. leukocytes, vascular endothelia, mucosal epithelia), which have been studied for their capacity to modulate inflammation (26). Second, murine models of inflammation provide evidence for adenosine receptor signaling as mechanism for regulating inflammatory response in vivo (27). Third, hypoxia is a common feature of inflamed tissue (28) and is accompanied by significantly increased levels of adenosine (29-31). At present, the exact source of adenosine is not well defined.

The present studies address the contribution of vascular endothelia to extracellular ATP elevations during conditions of hypoxia. While extracellular ATP levels are generally elevated during limited oxygen availability (34), the results from our studies point out that endothelial ATP release is actually attenuated by hypoxia exposure, suggesting that other cellular sources (e.g. platelets, red blood cells or inflammatory cells) (222, 233, 251, 252) must account for extracellular ATP elevations during limited oxygen availability. Extracellular ATP liberated during hypoxia and inflammation can signal direct on puringergic receptors or can activate adenosine receptors following regulated phosphohydrolysis via hypoxia-induced CD39 ectopyrase and CD73 ecto-5´-nucleotidase. In contrast to the present studies, a previous study of endothelial ATP release during hypoxia found increased extracellular ATP release from pulmonary endothelia following hypoxia exposure (34). Why these results are different from the present studies is currently unclear. A possible explanation may involve the fact, that pulmonary endothelia that were used in the studies by Gerasimovskaya et al. may be different in their responses to hypoxia than vascular endothelial cells that were used in the present studies (e.g. HMEC-1). As such, baseline levels of ATP release measured in the present studies of HMEC-1 were close to 100 nM, whereas baseline ATP levels measured by Gerasimoskaya et al. in pulmonary endothelia were approximately 0.2 nM. Moreover, differences in the dynamics of extracellular ATP metabolism in different cellular models or hypoxic conditions could also contribute to the differences between both studies.

The findings from the present studies highlight the role of extracellular ATP release as part of a crosstalk pathway between different cell-types during conditions of hypoxia. The fact that endothelial ATP release is attenuated

following hypoxia exposure suggests that other cellular components of the vasculature (e.g. smooth muscle cells, fibroblasts, platelets, red blood cells or inflammatory cells) (222, 233, 251, 252) have to account for hypoxia-associated increase in extracellular ATP. For example, non-resident cells like PMN that participate in an endothelial-neutrophil dependent crosstalk may therefore represent an important source of extracelluar ATP (36).

During episodes of hypoxia and inflammation PMN move into the underlying tissues by initially passing between endothelial cell (TEM, transendothelila migration). TEM is particularly prevalent in inflamed tissues, but also occurs a natural process of leukocyte mobilization (e.g. bone marrow extravasation). Understanding the biochemical details of leukocyte-endothelial interactions is currently an area of concentrated investigation. Detailed studies have revealed that the process of leukocyte TEM entails a concerted series of events involving intimate interactions of series of leukocyte and endothelial glycoproteins that include selectins, integrins, and members of the immunoglobuline supergene family (273, 274). Histological studies of TEM reveal that PMN initially adhere to endothelium, move to nearby interendothelial junctions via diapedesis, and insert pseudopodia into the interendothelial paracellular space (12). Succesful TEM is accomplished by temporary PMN self-deformation with localized widening of the interendothelial junction. Particularly during episodes of inflammation TEM creates the potential of disturbance of vascular barrier and concomitant loss of extravascular fluid and resultant edema. However, several innate mechanisms have been described to dampen fluid loss during PMN-endothelial interactions (64). For example, following TEM, adjacent endothelial cells appear to reseal leaving no residual interendothelial gap (12). Several crosstalk pathways have been identified to protect endothelial permeability

during inflammation and hypoxia and to dampen excessive fluid loss into the interstitium. Such innate protective pathways share the common strategy to increase intravascular adenosine concentrations and adenosine signaling with the inflamed or hypoxic vasculature (8, 35, 48, 64).

PMNs function as a first line of cellular response during acute inflammatory episodes (28). Current studies reveal that activated PMN derived soluble mediators like adenosine and AMP are capable to increase the endothelial barrier and function via activation of $AdoRA_{2B}$ adenosine receptors (22). In addition, previous reports have suggested that PMNs may also release ATP during conditions of inflammation or hypoxia and such ATP is coordinately hydrolyzed to adenosine at the endothelial cell surface particularly under hypoxic conditions by induced CD39 and CD73 (35). Studies also pursued mechanisms and functional consequences of ATP release from activated PMN. Several mechanisms for ATP release have been proposed, including direct transport through ATP-binding cassette (ABC) proteins, vesicular release, as well as gap junctions (221). Gap junctions are composed of connexin protein subunits. Six connexin subunits can form a hemichannel (connexon). The connexin gene family comprises 21 members in humans. In the vascular wall four connexins (Cx37, Cx40, Cx43 and Cx45) have been detected that varies depending on the cell type and the compartement. Recently studies showed for the first time a role of Cx43 on the surface of PMNs in releasing ATP from inflammatory cells during activation (222). Confirmatory studies in inducible Cx43-deficient mice showed that Cx43 expression correlated with PMN ATP release.These studies demonstrate nucleotide liberation at sites of acute inflammation by PMN, and identify Cx43 dependent ATP release as a central

part of an innate inflammatory response controlling adenosine-dependent endothelial function.

The present studies address molecular mechanisms involved in endothelial ATP release. These studies point also towards a critical role of Cx43 in endothelial-dependent ATP release. Moreover, endothelial hypoxia results in transcriptional repression of Cx43 in conjunction with increased phosphorylation status of Cx43- ser368, thereby resulting in hypoxia-associated attenuation of ATP-release.

Many connexins have been shown to be phosphoproteins (Cx31, Cx32, Cx37, Cx40, Cx43, Cx45, Cx50 and Cx56). Phosphorylation of Cx43 is associated with a functional change of Cx43 from an open to a closed state (163). Under normal conditions, Cx43 is phophorylated on multiple serines. Consistent with our findings, previous studies indicated that Cx43 phosphorylation can be modulated by inflammation and hypoxia. For instance, dephosphorylation of Cx43 and uncoupling of myocardial gap junctions occurs during myocardial ischemia. Under such circumstances, Cx43 may be reversibly dephosphorylated and rephosphorylated during hypoxia and reoxygenation dependent on fluctuations in intracellular ATP content (275). Moreover, several studies have implicated a role for Cx43 in cardioprotection by ischemic preconditioning, insomuch as protection by ischemic preconditioning is lost in cardiomyocytes and hearts of heterozygous Cx43-deficient mice (276). It is tempting to speculate that the role of Cx43 as ATP channel may also be involveld in cardioprotection by ischemic preconditioning. In fact, this may point to a clinical role of Cx43-dependent ATP release in myocardial ischemia. Thus, PMN-dependent ATP release could represent an important substrate for

nucleotidase-dependent extracellular adenosine generation during cardioprotection by ischemic preconditioning.

Recently studies showed that pannexins also can form functional intercellular channels. Pannexins (Panx) are orthologs of the invertebrate innexins. Vertebrates express both connexins and pannexins. Three pannexin are known, pannexin 1,2 and 3. Despite the lack of significant sequence homology, strong similarities exist at the structural and functional level with canonical gap junction proteins. Expression of pannexins in paired oocytes reveald that at least one of them, Panx 1, forms functional intercellular channels. Panx 1 exhibits hemichannel acitivity in single oocytes, i.e. membrane channel is formed by allowing flux of ions and larger molecules, like ATP, across the cell membrane (95). Studies revealed that pannexins are mechanosensitiv and highly permeable to ATP (96). Support for an involvement of pannxin 1 in ATP release comes from erythroytes. Erythrocytes do not contain Cx43, but pannexin 1 and when exposed to hypoxia erythrocytes release ATP (97).

The findings from these studies focus on another interesting aspect of ATP release and should be examined more exactly within the next years.

Activated plateles comprise an additional source for extracellular adenine nucleotides (37, 220). Metabolism of the adenine nucleotides is crucial in limiting excessive platelet aggregation and thrombus formation (40).

Extracellular ATP can be rapidly metabolized to adenosine by hypoxia-induced ectonucleotidases (CD39, ecto-apyrase, conversion of ATP to AMP and CD73, ecto-5’-nucleotidase, AMP to adenosine) expressed on the endothelial surface, thereby contributing to endothelial adaptation to hypoxia.

CD39 and CD39-like molecules are widely distributed in most tissues and have various orientations within the cells (35, 51). CD39 has been considered in the

modulation of platelet purinoreceptor activity by the sequential hydrolysis of extracellular ATP or ADP. Endothelilal CD39 plays a protective, thromboregulatory role in restricting the size of the hemostatic plug by limiting excessive platelet aggregation (39, 40). Indeed, excessive platelet accumulation and recruitment can be treated with soluble forms of CD39 (42, 43). Moreover, a thromboregulatory role could be demonstrated in a model of stroke or ischemia-reperfusions injury, where damage in CD39-null mice was readily treated with soluble forms of CD39/apyrase (44). Targeted disruption of CD39 resulted in prolonged bleeding and increased vascular leak, suggesting a dual role of for ATP metabolism by CD39 in modulating both hemostatic and thrombotic reactions (45).

CD73 is a membrane bound glycoprotein. Endothelial cells of many origins express constitutive CD73. The primary function attributed to endothelial CD73 has been catabolism of extracellular nucleotides, although CD73 may also mediate lymphocyte binding under some circumstances (206).

In fact, different studies have shown that during conditions of hypoxia or ischemia, the main source of extracellular adenosine stems from phosphohydrolysis of precursor molecules, particularly ATP (35, 237-240). Studies demonstrated that hypoxia promotes the induction of CD39 for the initiation of ATP phosphohydrolysis to adenosine (38) and revealed also that increased activity of endothelial CD39, as seen after hypoxic exposure, is necessary for the barrier protective influence of ATP.

Similarly, these studies revealed that additional metabolic and signaling molecules (CD73 and $AdoRA_{2B}$) are coordinately induced by hypoxia. Previous studies suggest that CD73 is a key component of a protective pathway to maintain barrier function in epithelial (48) and endothelial (35). For example,

administration of the selective ecto-5´-NT inhibitor APCP to mice by gavage significantly increased intestinal epithelial permeability (48). Furthermore, the barrier-promoting function of ATP released from activated neutrophils in vitro was found to be dependent on ATP hydrolysis to adenosine via the coordinated action of CD39 (Figure 8). In vivo experiments in mice deficient in extracellular adenosine generation (cd73$^{-/-}$ or cd39$^{-/-}$ mice) (35, 51) revealed dramatic increases in vascular leakage and pulmonary edema when mice were exposed to ambient hypoxia (8% of oxygen over 4h) (35, 51).

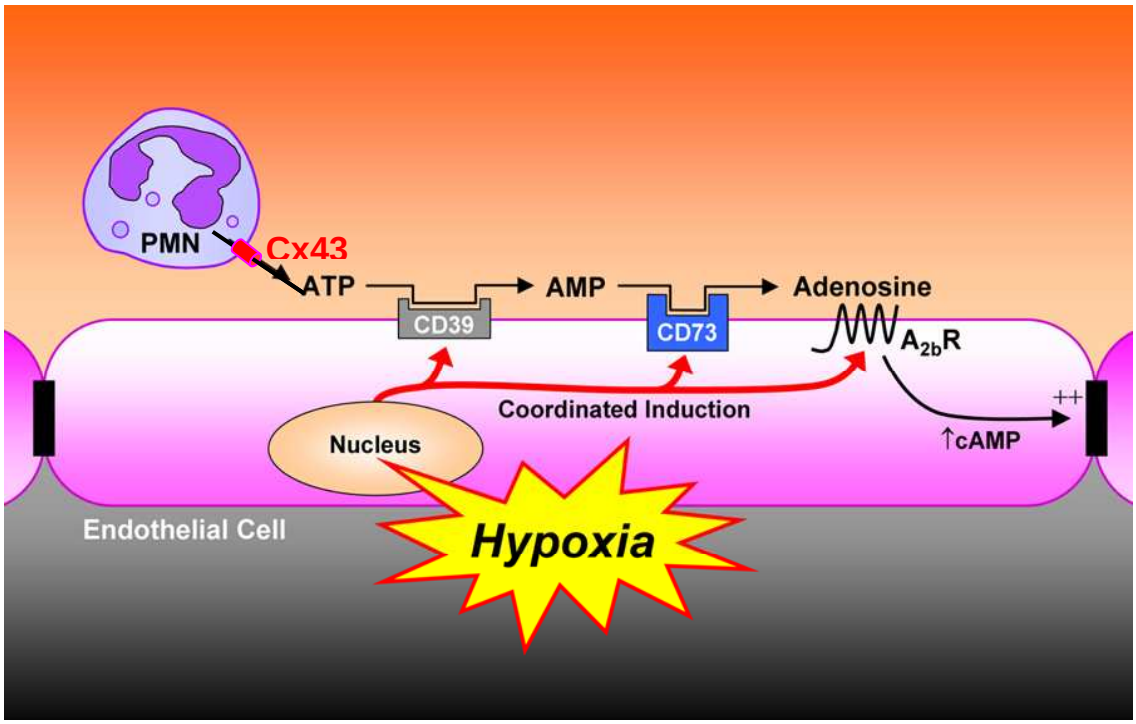



***Figure 8:*** *Model of PMN – endothelial crosstalk by extracellular nucleotides.*

*At sites of hypoxia or ongoing inflammation, activated PMN provide an extracellular source of ATP through membrane Cx43 hemichannels. ATP released in this fashion is metabolized through two enzymatic steps and results in the liberation of extracellular adenosine. Adenosine generated through this pathway is available for activation of surface endothelial adenosine receptors,*

*particularly the AdoA2BR. Diminished oxygen supply (hypoxia) at sites of inflammation coordinates the induction of CD39, CD73 and AdoA2BR. At such sites, post-receptor increases in intracellular cyclic AMP result in enhanced barrier function. This biochemical crosstalk mechanism may provide an innate mechanism to preserve vascular integrity and attenuate vascular leak (222).*

Adenosine can activate one of four types of G-protein coupled, seven transmembrans spanning adenosine receptors ($AdoRA_{1A}$, $AdoRA_{2A}$, $AdoRA_{2B}$, $AdoRA_{3A}$). Adenosine receptors are expressed on a wide variety of cells. Many cell types have been shown to express more than one isoform of the receptor. Likewise, activation of surface adenosine receptors has been shown to regulate diverse physiologic endpoints. A recent study of vascular responses to hypoxia compared gene-targeted mice for each individual adenosine receptor showing a barrier-protective role of signaling through the $AdoRA_{2B}$ in attenuating vascular leakage during hypoxia (247). Moreover, these studies found resuscitation of endothelial barrier defects associated with hypoxia by $AdoRA_{2B}$ agonist treatment. Recent studies show that transendothelial migration of PMNs can be attenuated by adenosine activation of $AdoRA_{2B}$ (277). Similarily, targeted disruption of the $AdoRA_{2A}$ receptor is associated with dramatically increased inflammatory reactions to subthreshold stimuli in an animal model (27).

Once generated into the extracellular milieu, adenosine can also rapidly cleared through passive or active uptake by nucleoside transporters. Nucleoside transporters comprise two widely expressed families, the equiliberative nucleoside transporters (ENTs) (diffusion-limited channels) and concentrative nucleoside transporters (CNTs) (sodium-dependent transporters), respectively,

expressed on a variety of cell types (278, 279). Because of their anatomic position at the blood-tissue interface, vascular nucleoside transporters are in an ideal position to influence vascular nucleoside levels, particularly adenosine, which among others plays an important role in tissue protection during acute injury. Recent studies suggest that the predominant functional nucleoside transporters in the vascular endothelium are ENT1 and ENT2. ENT1 and 2 are bidirectional transporters functioning as diffusion limited channels for transmembrane nucleoside flux. Previous studies have suggested that vascular adenosine transport during hypoxia is predominately inward (209), thereby terminating extracellular adenosine signaling. However, more recent studies show that the expression of ENT1 may be trancriptionally regulated by hypoxia (215), thereby functioning to fine tune extracellular levels of adenosine. Further studies revealed that endothelial and epithelial ENT1/ENT2 gene expression and function are attenuated by hypoxia, and that this regulatory cirucuit maps to hypoxia inducible factor 1 (HIF-1)-mediated repression of ENT expression (245). These results identify repression of ENT as a mechanism to elevate extracellular adenosine during hypoxia.

As demonstrated above, hypoxia and inflammation is an area of intensive investigation and an interplay of many facors. Our studies pursue the contribution of vascular endothelia to extracellular ATP release unter hypoxic conditions. Taken together, the present studies define an important contribution of endothelial Cx43 to extracellular ATP release and reveal transcription and phosphorylation dependent attenuation of extracellular ATP release from vascular endothelia during conditions of limited oxygen availability

# 7. SUMMARY

Extracellular ATP is an important signaling molecule for vascular adaptation to limited oxygen availability (hypoxia). Here, we pursued the contribution of vascular endothelia to extracellular ATP release under hypoxic conditions.

We gained first insight from studying ATP release from endothelia (HMEC-1) pre-exposed to hypoxia. Surprisingly, we found that ATP release was significantly attenuated following hypoxia exposure (2% oxygen, 22±3% after 48h). In contrast, intracellular ATP was unchanged. Similarly, lactate-dehydrogenase release into the supernatants was similar between normoxic or hypoxic endothelia, suggesting that differences in lytic ATP release between normoxia or hypoxia are minimal. Next, we used pharmacological strategies to study potential mechanisms for endothelial-dependent ATP release (eg, verapamil, dipyridamole, 18-alpha-glycyrrhetinic acid, anandamide, connexin-mimetic peptides). These studies revealed that endothelial ATP release occurs – at least in part - through connexin 43 (Cx43) hemichannels. A real-time RT-PCR screen of endothelial connexin expression showed selective repression of Cx43 transcript and additional studies confirmed time-dependent Cx43 mRNA, total and surface protein repression during hypoxia. In addition, hypoxia resulted in Cx43-serine368 phosphorylation, which is known to switch Cx43 hemi-channels from an open to a closed state. Taken together, these studies implicate endothelial Cx43 in hypoxia-associated repression of endothelial ATP release.

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