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# EVALUATION OF EXTRACEL- LULAR NUCLEOTIDE TURNO- VER IN VASCULAR ENDO- THELIAL CELLS UNDER NOR- MAL AND HYPOXIC STATES



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## EVALUATION OF EXTRACELLULAR NUCLEOTIDE TURNOVER IN VASCULAR ENDOTHELIAL CELLS UNDER NORMAL AND HYPOXIC STATES

This bachelor's thesis was commissioned by Sirpa Jalkanen's research group which is part of the Faculty of Medicine in the University of Turku. The research group is focused on studying leukocyte trafficking and adhesion.

Besides working as intracellular energy molecules, nucleotides such as ATP and ADP also act as extracellular signaling molecules in various tissues and organs. Especially ATP is involved in several physiological and pathological functions serving for instance as a pro-inflammatory agent. Cells release purines and pyrimidines to the extracellular milieu as a result of various stress conditions such as decreased oxygen level (hypoxia). Hypoxia is known to be linked with the formation of new blood vessels (angiogenesis), thereby contributing to the increased incidence of cancer.

The aim of this thesis was to study the effects of acute hypoxia (4-24 h; 1% O<sub>2</sub>) on human umbilical vein endothelial cells (HUVEC). In addition, the effects of one hour re-oxygenation after hypoxia were studied. Results were compared with cells cultured under normal oxygen supply at body temperature.

The activities of the nucleotide-converting ecto-enzymes were determined with a thin layer chromatography (TLC) using <sup>3</sup>H- labelled substrates. The tyrosine kinase phosphorylation status of the cells was studied by Western Blot analysis and immunofluorescence staining. The concentrations of extracellular ATP and ADP were determined with a bioluminescent approach based on luciferin-luciferase reaction.

According to the results, acute hypoxia did not have any significant effect on the extracellular nucleotide levels while the phosphorylation status of HUVECs was diminished. Interestingly, endothelial ecto-nucleotidase activities were slightly up-regulated during hypoxic exposure and in addition, the counteracting ATP-regenerating pathway was concurrently activated.

### KEYWORDS:

Hypoxia, HUVEC, nucleotide turnover

Camilla Tallgrén

# SOLUN ULKOPUOLISTEN NUKLEOTIDIEN MUUNTUMISEN ARVIOINTI VASKULAARISISSA ENDOTEELISOLUISSA NORMAALISSA JA HYPOKSISSA TILASSA

Tämän opinnäytetyön toimeksiantajana oli Sirpa Jalkasen tutkimusryhmä, joka on osa Turun yliopiston lääketieteellistä tiedekuntaa. Tutkimusryhmä on keskittynyt tutkimaan erityisesti leukosyytiliikennettä ja adheesiota.

Solun ulkopuoliset nukleotidit, kuten ATP ja ADP toimivat sekä solun sisäisinä energiamolekyyleinä, että solun ulkopuolisina signaalimolekyyleinä useimmissa kudoksissa ja elimissä. Etenkin ATP on osallisena useissa fysiologisissa ja patologisissa tehtävissä toimien esimerkiksi tulehdusta edistävänä tekijänä. Solut vapauttavat puriineja ja pyrimidiinejä solun ulkopuoliseen tilaan mm. erilaisten stressitilojen, kuten vähentyneen hapen määrän eli hypoksian vaikutuksesta. Useissa tutkimuksissa hypoksian on todettu olevan yhteydessä uusien verisuonten muodostumiseen eli angiogeneesiin ja siten myös syövän syntyyn.

Tämän opinnäytetyön tarkoituksena oli tutkia akuutin hypoksian (4-24 h; 1% O<sub>2</sub>) seurauksia ihmisen napanuoran laskimon endoteelisoluissa (HUVEC). Myös hypoksian jälkeisen toipumisen vaikutuksia tutkittiin (1 h). Tuloksia verrattiin solun normaaliin hapensaantiin ruumiinlämpötilassa.

Nukleotideja muuntavien ektoentsyymien aktiivisuudet määritettiin <sup>3</sup>H- leimattujen substraattien avulla käyttäen ohutlevykromatografiaa (TLC). Solujen tyrosiinikinaasifosforylaatiostatusta analysoitiin Western Blot -analyysin ja immunofluoresenssivärjäysten avulla. Solun ulkopuolelle vapautuvien ATP:n ja ADP:n konsentraatiot määritettiin bioluminesenssipohjaisella menetelmällä, joka perustuu lusiferiini-lusiferaasireaktioon.

Tulosten mukaan akuutilla hypoksialla ei ollut merkittäviä vaikutuksia solun ulkopuolisiin nukleotidipitoisuuksiin. Lisäksi solujen fosforylaatio väheni akuutin hypoksian vaikutuksesta. Mielienkiintoista oli, että endoteelin ekto-nukleotidaasiaktiivisuudet lisääntyivät hieman hypoksian seurauksesta ja ATP:a muodostavan mekanismin todettiin olevan aktivoitunut.

## ASIASANAT:

HUVEC, Hypoksia, nukleotidien muuntuminen

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## **LIST OF ABBREVIATIONS**

ADA= Adenosine deaminase

Ado= Adenosine

AK= Adenylate kinase

BSA= Bovine serum Albumin

EC= Endothelial cell

ECL= Enhanced chemiluminescence

ECM=Endothelial cell matrix

FGF= Fibroblast growth factor

HIF= Hypoxia inducible factor

HRP= Horse radish peroxidase

HUVEC= Human umbilical vein endothelial cells

IL-1= Interleukin-1

MAPK= Mitogen activated protein kinase

NDPK= Nucleoside diphosphate kinase

NF $\kappa$ B= Nuclear factor  $\kappa$ B

PBS= Phosphate buffered saline

(p)FAK= (Phosphorylated) focal adhesion kinase

PVDF= Polyvinylidene fluoride

RT= Room temperature

SDS-PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMC= Smooth muscle cell

TBS= Tris buffered saline

TLC= Thin layer chromatography

TEMED= N, N,N',N'-Tetramethylenediamine

TNF $\alpha$ = Tumour necrosis factor  $\alpha$

VEGF= Vascular endothelial growth factor

# 1 INTRODUCTION

Oxygen delivery to the cells may be compromised by tissue damage or be inadequate due to the the increased metabolic demands, leading to hypoxia. Probably the most important regulator of cell responses to hypoxia is the hypoxia inducible factor (HIF). (Walmsley *et al*, 2008.) Studies suggest that different types of stress conditions including hypoxia relate to the elevated extracellular ATP levels in the vascular wall. Extracellular purine and pyrimidine nucleotides, such as ATP, act as a ligand for purinergic receptors (P2Y and P2X) and they are believed to be important regulators of vascular functions contributing to the development of vascular diseases. (Gerasimovskaya *et al*, 2010.) Purinergic signaling is linked to various pathological and physiological processes (Wall *et al*, 2008). The wide tissue distribution of purinergic receptors together with the expression of various receptor subtypes encourages the importance of purinergic signaling in regulation of various cell functions. (Gerasimovskaya *et al*, 2007, 2010.)

In this thesis, the objective was to study the effects of acute hypoxia on the purinergic signaling cascade in endothelial cells isolated from human umbilical vein. The connections between hypoxia, angiogenesis and purinergic signaling, which are prominent features in this thesis, are discussed in more detail in the following chapters.

## 1.1 Hypoxia as a model of inflammation and induction of angiogenesis

### 1.1.1 Angiogenesis

Hypoxia has been shown to cause angiogenesis, formation of new capillaries from existing blood vessels. Blood vessel growth and regeneration are vital for human life since blood vessels form the largest network in our body. Blood vessels include veins, arteries and capillaries. The smallest capillaries are formed only by endothelial cells (EC), but medium sized veins and large vessels are comprised of smooth muscle cells (SMCs) and pericytes. Capillary sprouts form new vessels, eventually creating a hollow tube. (Kuldo *et al*, 2005)

Endothelial cells differentiate in the embryonic state or in the postnatal state by various mechanisms. Blood vessel growth is a highly regulated process, and angiogenesis is part of the normal process for example in embryonic development. In addition, angiogenesis is also present during various pathological conditions involving hypoxia, inflammation, cancer and asthma. (Gerasimovskaya *et al*, 2007.)

Endothelial cells have four main functions, of which the first is working as a semipermeable barrier for soluble molecule transport. Besides, endothelial cells maintain haemostatic balances, recruit leukocytes into underlying tissue and participate actively in neovascularisation. (Kuldo *et al*, 2005)

### 1.1.2 Hypoxia

All aerobic organisms need oxygen for the generation of adenosine triphosphate (ATP), which is a vital molecule for a large number of biochemical processes. In normal state, oxygen uptake and consumption are in balance, but when the oxygen supply to the cell is insufficient compared with the consumption, a hypoxic state follows. The demand of oxygen varies by the cell type and some cells have been reported to be hypoxic in their normal state, for example the thymus and bone marrow niche. The normal oxygen level in cells vary between 2 and 12 kPa. (Walmsley *et al*, 2008.)

Several reasons, for instance an inflammation, tumour growth, great physical stress or high altitudes may lead to the hypoxic state (Walmsley *et al*, 2008). Hypoxia is also present in the wound healing process, where the increased oxygen demand leads to reduced oxygen concentrations in tissue, wound healing fibroblast proliferation besides collagen synthesis and VEGF activation (Rodriguez *et al*, 2008).

#### 1.1.2.1 Hypoxia inducible factor

Oxygen demanding species express HIF, a transcription factor, which is one of the most important effectors during hypoxia, regulating short- and long-term adaptations of cells to hypoxia (Kuldo *et al*, 2005, Walmsley *et al*. 2008). HIF is

a heterodimeric protein composed of  $\alpha$ - and  $\beta$ -subunits. The  $\alpha$ -subunits are HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ , which all have their own expression tissues. HIF-1 $\alpha$  activation may not result only from hypoxic conditions, but also from shear stress. (Walmsley *et al*, 2008.) With the activation of HIF-1, tissues try to regulate oxygen levels in cells. Extracellular ATP, for its part controls purinergic receptors and nitric oxide (NO) is released leading to vasodilation, which allows greater perfusion and this way endothelial cells also involved in blood pressure control. (Kuldo *et al*, 2005) HIF-1 activation leads also to induced erythropoiesis, angiogenesis and increased vascular permeability (Manalo *et al*, 2005). The vascular endothelial growth factor (VEGF) is a growth factor that affects vascular development. HIF-1 upregulates the production of VEGF and many other growth factors (Kuldo *et al*, 2005).

Growth factors and cytokines are released from the extracellular matrix after different stimuli or produced as soluble extracellular glycoproteins. Cytokines and growth factors affect the activation status of endothelial cells by receptor-mediated intracellular signalling. Because of the cytokine and growth factor activation in chronic inflammation, endothelial cells participate in angiogenesis and recruit immune cells from blood circulation to the underlying tissue. Immune cell recruitment is one of the four main functions of endothelial cells. Besides, endothelial cells maintain haemostatic balances, participate actively in neovascularisation and act as a semipermeable barrier for soluble molecule transport. Several growth factors are related to angiogenesis and inflammation, for instance the fibroblast growth factor (FGF), interleukin-1 (IL-1), the vascular endothelial growth factor (VEGF) and the tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), the latter being able to activate various inflammation causing pathways in ECs. Triggering of the receptors leads to for instance activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B), mitogen activated protein kinase (MAPK) and nitric oxide production and eventually to the expression of inflammation process controlling adhesion molecules and other genes. NF- $\kappa$ B is a key regulator of transcription in hypoxia associated inflammation. (Kuldo *et al*, 2005.)

The co-activation of VEGF receptor-2, expression of VCAM-1 and monocyte recruitment are associated with ATP- and UTP-induced stimulation of P2Y2 receptor in endothelial cells. Extracellular ATP and UTP have also proved to stimulate the DNA synthesis in vascular ECs, SMCs and adventitial fibroblasts. This implies to a link between angiogenesis, purinergic signalling and inflammatory responses. (Gerasimovskaya *et al*, 2007 & 2010.)

## 1.2 Characterization of purinergic signalling cascade with particular emphasis on nucleotide-converting pathways

The model of purinergic signalling was first proposed by G. Burnstock in 1972 (Burnstock, 1972). Purines are heterocyclic aromatic organic compounds that have a pyrimidine ring connected to the imidazole ring. Since 70s, the role of nucleotides as signalling molecules has been well established and generally acknowledged. The signaling roles of the nucleotides include the regulation of epithelial cell responses, oxygen delivery, immune responses, activation and aggregation of platelets at the sites of vascular injury, blood flow distribution, gastrointestinal and liver functions, just to mention a few. (Burnstock, 2007)

It is noticed that besides acute signalling events, purines may have an important long-term role in cell proliferation, cell growth, wound healing, the induction of apoptosis and anticancer activity. According to current concepts, nucleotide turnover includes four steps. First ATP and ADP are released at nanomolar concentrations and trigger signalling events via purine and pyrimidine receptors. Nucleotides are then broken down by nucleotidases. The resulting nucleotide-derived adenosine binds to its own nucleoside-selective receptors and is finally further deaminated on the cell surface or transported into the cell via specific nucleoside transporters. (Yegutkin, 2008.)

The most important purines are adenosine and its high energy phosphorylated derivatives (Yegutkin, 2008). ATP was first discovered in 1929 by two separate laboratories but not much interest was given to the molecule then. The complete structure of ATP was established in 1945 and ever then this multifunctioning nucleotide has gained more attention (Lythogoe and Todd, 1945). ATP is

an important energy source for the body. Intracellular ATP works in energy-requiring processes such as active transport, cell motility and biosynthesis. Extracellular ATP, instead has a great role as a signalling molecule (Yegutkin, 2008.)

### 1.2.1 Nucleotide releasing mechanisms

Cells can release nucleotides because of cell lysis but also by non-lytic mechanisms. Various excitatory tissues such as nerve terminals and circulating platelets store ATP and ADP with other neurotransmitters and extracellular mediators in specialized granules. The content of the granules is released to the extracellular milieu via  $\text{Ca}^{2+}$ -dependent exocytosis. There are also non-excitatory tissues such as epithelial and endothelial cells releasing ATP under mechanical and other stimuli including hypoxia. Cells are proposed to release nucleotides by electrodiffusion, nucleotide diffusion through ATP-binding cassette transporters or cargo-vesicle trafficking and exocytotic secretion (Figure 1.). Besides ATP and ADP, adenosine monophosphate (AMP) and adenosine can also appear in the extracellular space either via direct secretion from the cell or as a result of stepwise dephosphorylation of extracellular ATP and ADP (Yegutkin, 2008). Nucleotide-releasing pathways are summarized in Figure 1.

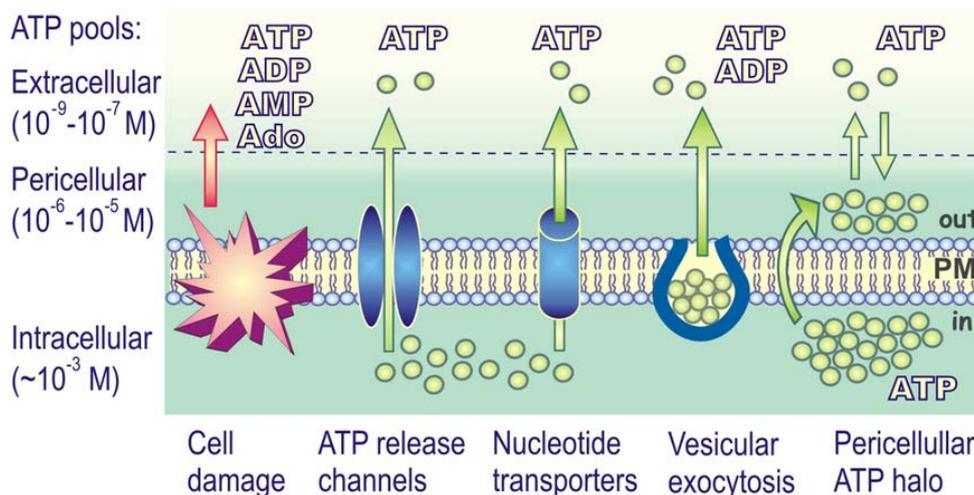


Figure 1. Nucleotide releasing mechanisms. Nucleotides can be released into the extracellular milieu during cell damage but also via various molecular mechanisms including electrodiffusional movement through ATP release channels, vesicular exocytosis and diffusion by nucleotide-specific transporters. ATP may also form an ATP halo where the ATP concentration is within the micromolar range. (Yegutkin, 2008.)

### 1.2.2 Nucleotide converting ectoenzymes

The released ATP and ADP are inactivated to AMP via ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) activity. AMP is further converted to anti-inflammatory adenosine via the ecto-5'-nucleotidase (5'-NT) (known also as CD73) reaction (Yegutkin *et al*, 2003, Gerasimovskaya *et al*. 2010). E-NTPDase family consists of eight ectoenzymes with molecular masses of 70-80 kDa which hydrolyse nucleoside tri- and diphosphates but not nucleoside monophosphates (Gerasimovskaya *et al*, 2010). These ectoenzymes need millimolar concentration of Ca<sup>2+</sup> and Mg<sup>2+</sup> for maximal activity (Yegutkin, 2008). Four of them are expressed on the cell surface (NTPDase 1,2,3,8), two of them are located intracellularly and two of them are soluble enzymes which can be secreted outside the cell (Gerasimovskaya *et al*, 2010).

Mammalian ecto-5'-nucleotidases are composed of two glycoprotein subunits with molecular masses of 60-70 kDa. Seven different human 5'-nucleotidases have been found to date. (Yegutkin, 2008.) 5'-nucleotidases are hydrolytic en-

zymes which catalyze the hydrolysis of AMP and other nucleoside monophosphates into forms in which they can be used for metabolic needs of cells, for example  $\text{AMP} \rightarrow \text{Ado}$  (Li *et al*, 2006). Ecto-5`NT is expressed in various different tissues including the brain, liver and vascular endothelium of large vessels (Yegutkin, 2008). It is anchored in the plasma membrane by glycosylphosphatidylinositol (Gerasimovskaya *et al*, 2010). Besides functioning as an enzyme CD73 also mediates cell adhesion to the matrix, cell adhesion to other cells and induces intracellular signaling (Salmi and Jalkanen, 2006).

Various families of nucleotide breaking ecto-enzymes have been described in literature but these two enzyme groups are proposed to be the major regulators of purinergic signaling. Also alkaline phosphatases and ecto-nucleotide pyrophosphatase phosphodiesterases (E-NPP-family members) contribute to the inactivation of nucleotides (Yegutkin *et al*, 2003). The formed adenosine interacts with its own G-protein coupled receptors. Extracellular adenosine is transported into the cell or inactivated to inosine. Adenosine is deaminated to inosine catalyzed by adenosine deaminase (ADA). ADA is expressed in many lymphoid and non-lymphoid tissues. Purine nucleoside phosphorylase (PNP) is an enzyme which further phosphorylates (deoxy)guanosine and (deoxy)inosine to guanine and hypoxanthine. They are metabolised to stable end-product uric acid via xanthine. (Yegutkin, 2008.)

Along with the inactivation pathway, a scheme of backward nucleotide resynthesis via ecto-nucleotide kinase-mediated phosphotransfer reactions has been proposed ( $\text{AMP} \rightarrow \text{ADP} \rightarrow \text{ATP}$ )(Yegutkin *et al*, 2003). Intracellular enzymes adenylate kinase and nucleoside diphosphate kinase (AK and NDPK) are co-expressed on the surfaces of the endothelial cells controlling local nucleotide concentrations through backwarded ATP-regenerating pathway. Adenylate kinase catalyzes the reversible reaction of  $\text{ATP} + \text{AMP} \rightleftharpoons 2 \text{ADP}$ . NDPK is an enzyme which catalyzes transfer of  $\gamma$ -phosphate transfer from nucleoside 5`-triphosphates to nucleoside 5`-diphosphates and it requires divalent cations for example  $\text{Mg}^{2+}$ . (Yegutkin, 2008) Scheme of ectoenzymatic purine turnover is shown in Figure 2.

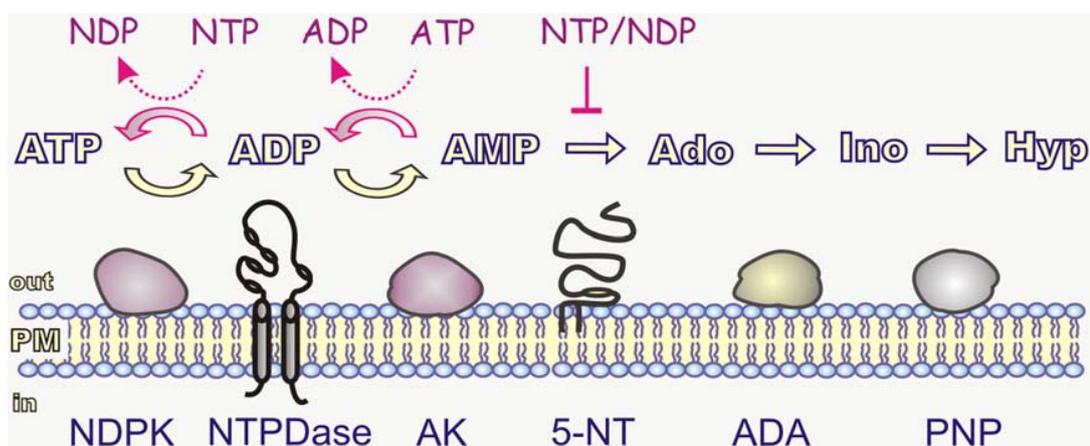


Figure 2. Ectoenzymatic purine turnover. Purines are sequentially inactivated by a network of purine-converting enzymes. Dashed arrows means an activatory mechanism and blunted lines shows inhibition. (Yegutkin, 2008)

### 1.2.3 Purine and pyrimidine receptors

Several purine and pyrimidine receptors are divided into P1 and P2 receptor subtypes (Yegutkin and Burnstock, 1998). The P1 receptors are selective for adenosine and the P2 receptors are activated by nucleotides such as ATP and ADP (Burnstock, 2007). The P2 receptors are further divided into two subfamilies and those receptor subfamilies are P2X and P2Y. The P2X receptors are ligand-gated channel receptors and there are seven receptor subtypes, P2X<sub>1</sub>-P2X<sub>7</sub>. In response to ATP, the P2X-receptors gate extracellular cations. The P2Y receptors are G-protein coupled receptors and they are divided into the G<sub>q</sub> coupled receptors P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub> and to the G<sub>i</sub> coupled receptors P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>. The G<sub>q</sub> coupled receptors activate phospholipase C- $\beta$ , inhibit adenylyl cyclase and regulate ion channels. G-protein coupled receptor subfamily P1(A) has four different receptor subtypes: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, A<sub>3</sub>. A<sub>1</sub> and A<sub>3</sub> receptors inhibit adenylyl cyclase, whereas A<sub>2A</sub> and A<sub>2B</sub> activate it. (Yegutkin, 2008.) All the major receptor subtypes are summarized in Figure 3.

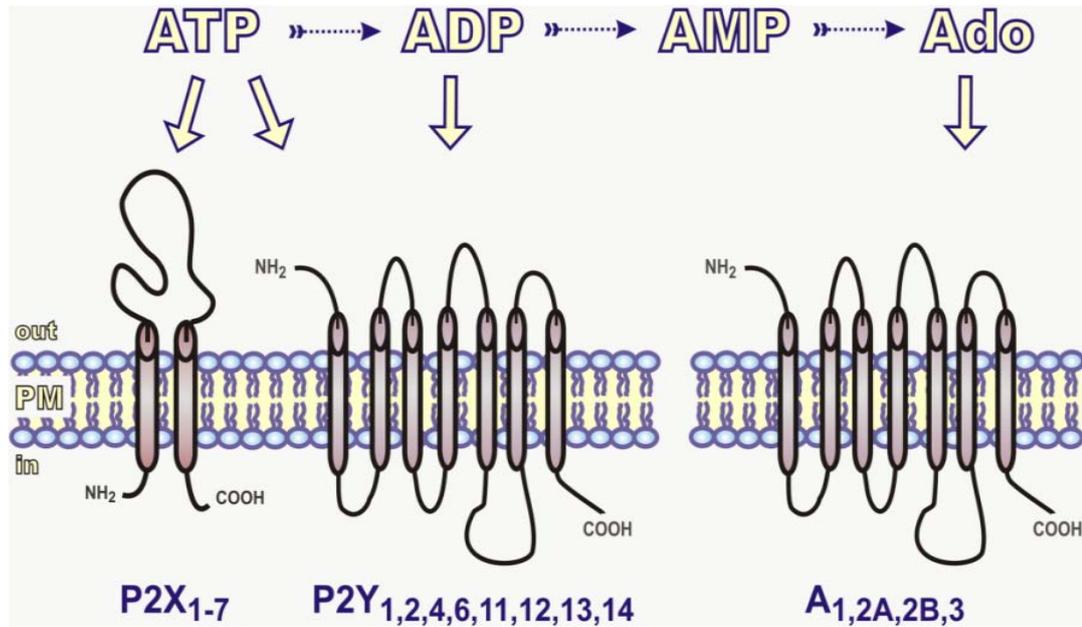


Figure 3. Purinergic signaling pathways. Signaling effects are mediated through seven ionotropic P2X receptors and eight metabotropic P2Y receptors. Adenosine acts at four different G-protein linked receptors. (Yegutkin, 2008)

## 2 PRINCIPLES AND RATIONALES OF THE EMPLOYED METHODS

### 2.1 Isolation and culturing of HUVEC

The objective of this thesis was to study the effects of short-term hypoxia (4-24 h; 1 % O<sub>2</sub>) on the purine metabolism in human umbilical vein endothelial cells (HUVEC). Primary HUVECs were isolated from human umbilical vein and kindly provided for me by our laboratory staff. My work included the subsequent culturing of the provided cells and the further performance of enzymatic and other assays under normoxic and hypoxic conditions, as specified below.

### 2.2 Acute hypoxia conditions

Hypoxic condition is caused by a lack of oxygen in cells. Acute hypoxia conditions for this study were created with a special hypoxic chamber (Biotrace Ruskinn Invivo<sub>2</sub> hypoxia workstation C400/CPlus) which is shown in Figure 4. Oxygen level in the hypoxic chamber is 1 %. Effects of hypoxia were examined for two different time periods, 4 h and 24 h. Cells were cultured in a hypoxic chamber for the desired time. Also the effects of the re-oxygenation wanted to be studied, so some of the samples were re-oxygenated in a normal + 37 °C incubator after culturing in the hypoxic chamber. Some of the cells were cultured in the presence of a 10 % fetal calf serum and some in an RPMI-1640 medium supplemented with 1% Bovine serum albumine (BSA).



Figure 4. Hypoxic chamber

### 2.3 Enzymatic assays

Radioenzymatic assays are used for the determination of enzyme activities and they are typically based on the conversion of the radiolabelled substrates to the labelled products. The substrate molecule is generally labelled with a certain isotope, for instance  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$  or  $^{125}\text{I}$ . Subsequently, this enables to determine quantities of a specific enzyme and to see changes in level or location of the molecule. Radiometric enzyme assays are widely used due to their high sensitivity and specificity. (Eisenthal and Danson, 2002, Alberts *et al*, 2002)

Thin layer chromatography (TLC) assay has shown to be one of the most diversified and suitable method for studying the adenine nucleotide catabolism, whereby it was chosen for our experiments. In the TLC assay, cells are incubated with an appropriate radioisotopic substrate followed by a separation with thin layer chromatography using nucleotide and nucleoside standard solutions for the identification of the end products. The order of nucleotide and nucleoside

standards is shown in Figure 6. (Yegutkin and Burnstock, 1998) Radiation can be measured with a scintillation counter, which detects the small flashes of light in a scintillation fluid induced by beta particles (Perkin Elmer, 2010 [Refered 14.4.2011]).

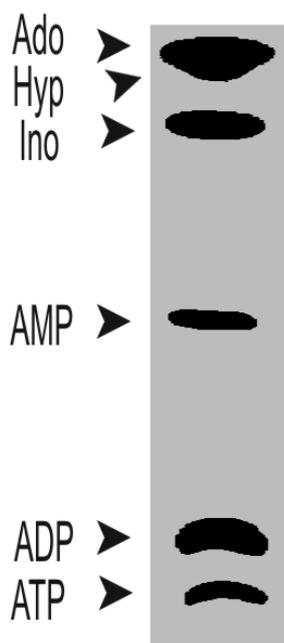


Figure 5. Order of nucleotides and nucleosides in the standard solution separated by TLC. The standard solution consists of adenosine (Ado), hypoxanthine (Hyp), inosine (Ino), adenosine monophosphate (AMP), adenosine di- and tri-phosphates (ADP and ATP), respectively. (Yegutkin *et al*, 2003)

Activities of three different enzymes ATPase, 5'-nucleotidase and adenylate kinase were determined and the enzyme activities were calculated using Formula 1. and were then expressed per  $10^6$  cells. Statistical analysis was done with the Microsoft Office Excell two-way student's t-test. p-values lower than 0.05 were considered as a statistically significant.

**Formula 1.**

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

Smpl = <sup>3</sup>H - Counts from the sample

Bl = <sup>3</sup>H - Counts from blank

[Subst] = Substrate concentration

Total = Total <sup>3</sup>H - Counts from a control

t = Incubation time

s = Volume added to TLC-plate (μl)

AV = Total reaction volume (ml)

**2.4 ATP and ADP measurement**

ATP assays were done according to the Perkin Elmers ATPLite™ Kit, which is based on the reaction of firefly luciferase. Luciferase catalyzes reaction between ATP and D-Luciferin, forming oxyluciferin, adenosine monophosphate, pyrophosphate, carbon dioxide and light. This chemical equation is shown below. The forming light can be measured with a luminometer and the ATP concentration is proportional to the emitted light. (Perkin Elmer, 2002 [Referred 4.2.2011])

**2.5 Protein measurement**

Protein amounts of the cells were determined with the Pierce BCA protein assay kit. This method is based on a reduction of Cu<sup>2+</sup> to Cu<sup>1+</sup> in alkaline envi-

ronment. In the first step of the colour formation reaction copper-ions chelate in an alkaline medium, containing a sodium potassium tartrate, with peptides that have three or more amino acid residues forming light blue coloured complex.

In the second step of the colour formation two bicinchoninic acid (BCA) molecules react with one reduced cuprous-cation, formed in the first step, and create a strong purple colour BCA-copper complex. Purple colour absorbs visible light that is measured with a spectrophotometer on a wavelength of 562 nm. Protein amount is proportional to absorbance. Used method is linear to BSA in a working range from 20 to 2000  $\mu\text{g/mL}$  and the detection limit of the method is 5  $\mu\text{g/mL}$ . (Thermo Fisher Scientific, 2011 [Refered 14.4.2011])

## 2.6 Immunofluorescence staining

Immunofluorescence is a technique used for the visualization of proteins or other antigens in cells or tissue sections using antibodies labelled with a certain fluorescent dye (fluorochrome), such as fluorescein isothiocyanate (FITC). There are two types of immunofluorescence staining methods: a direct immunofluorescence staining and an indirect immunofluorescence staining. In a direct method, primary antibody is labelled with a fluorescent dye and in an indirect method a secondary antibody labelled with fluorochrome, recognizes the primary antibody and conjugates with it. Both methods are shown in Figure 6. A stronger signal is achieved with the indirect method. Using secondary antibodies labelled with different fluorescein dyes enables the visualization of several antigens in one sample. Fluorochrome absorbs short wave length light and emits longer wave length light which can be detected with a fluorescence or a confocal microscope. (Alberts *et al*, 2002)

Every antibody has a unique binding site that recognizes only a specific target molecule. Preceding to the staining, cells are fixed to immobilize and preserve them. Molecules are cross linked to be in stabilized and locked-in positions permeable to staining reagents by paraformaldehyde, which forms covalent bonds with free amino groups of proteins. After fixing, cells are stained with an indirect method using several different antibodies. (Alberts *et al*, 2002)

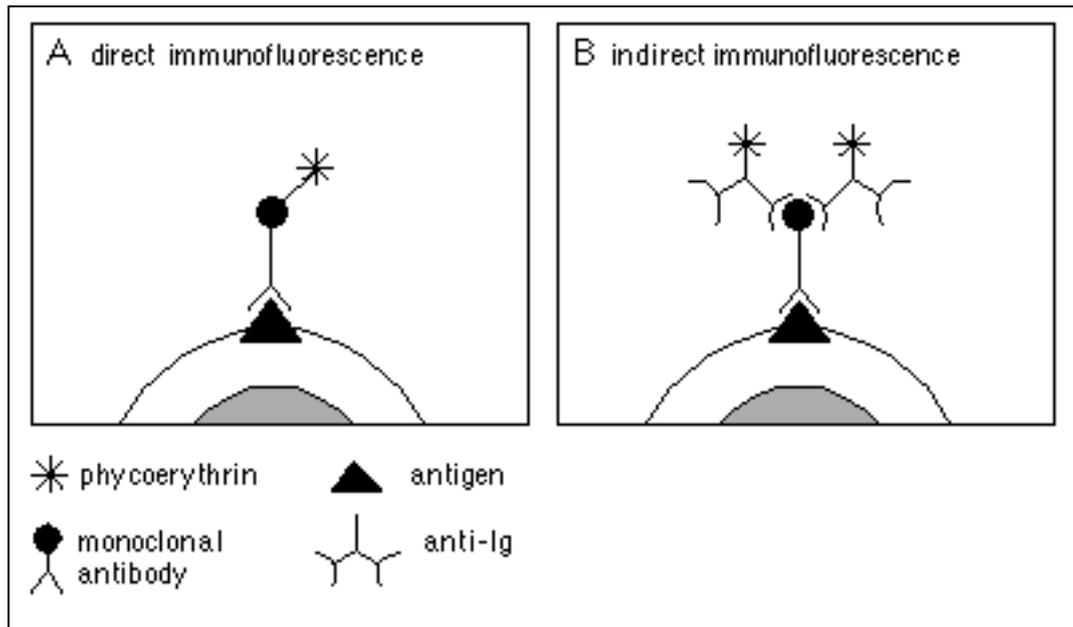


Figure 6. Direct and indirect immunofluorescence. (Zola, 2001 [Refered 4.2.2011])

## 2.7 SDS-PAGE and Western Blot

Western Blot is a technique which is used to analyze proteins based on their molecular weight. Proteins are first treated with sodium dodecyl sulfate (SDS), which charges proteins negatively when bound with these detergent molecules. SDS has such a strong negative charge that it covers protein's original charge also dissolving cell walls.  $\beta$ -mercaptoethanol changes proteins native conformation by breaking S-S linkages of the proteins. All the secondary, tertiary and quaternary conformations are broken and proteins are linear, which makes it possible for them to move only based on their sizes. When voltage is applied, the negatively charged proteins move towards the positive electrode. Proteins with the same size move through the gel similarly and are arranged by their molecular weight on the gel. (Alberts *et al*, 2002)

Proteins are then transferred from the gel onto a nitrocellulose or a polyvinylidene fluoride (PVDF) membrane by western blotting. Proteins detach from the gel when voltage is applied and travel to the membrane maintaining their organization which they had on the gel. Proteins are probed with primary antibodies

and secondary antibody, which binds to the primary antibody is used to create the signal. The horseradish peroxidase (HRP)-linked secondary antibody enables luminescence based detection. BSA, serum or skimmed milk are generally added to the blocking buffer to prevent a non-specific binding. Also over night incubation in a cold room reduces the non-specific binding. (Alberts *et al*, 2002)

The proteins were detected with the enhanced chemiluminescence (ECL) detection kit. ECL Detection kit's (Amersham) reagents together with HRP-labelled secondary antibody produce luminescence. When photographic film is placed against the nitrocellulose membrane and exposed to luminescence, the image of antibodies bound to the specific protein can be detected on the film.

## 2.8 Antibodies used in Western Blot and immunofluorescence analyses

The vertebrate immune system produces antibodies as a defence reaction to an infection. Antibodies, also known as immunoglobulins (Ig) are proteins which each of them having a unique binding site that recognizes specific antigens. Mammals can produce five different types of immunoglobulins IgA, ID, IgE, IgG and IgM. The simplest form of antibody is an Y-shaped molecule, that has two identical binding sites at the tips of both arms. (Alberts *et al*, 2002)

A cytoskeleton consists of microfilaments, composed of linear actin polymers, intermediate filaments made of vimentin, keratin, lamin or neurofilaments and microtubules consisted of  $\alpha$ - and  $\beta$ -tubulin. The cytoskeleton is required in cell motility, cell division, endo- and exocytosis and maintenance of cell shape, making it an important regulator of cell functions for most of the cell types. (Bogatcheva and Verin, 2008) Since cytoskeleton may be regulated during hypoxic exposure, we wanted to study the effects of hypoxia on cytoskeletal elements with anti-  $\beta$ -tubulin, anti-actin and anti-vimentin.

Integrins play a role in cell signaling mediating attachment between a cell and the tissue surrounding the cell. Integrins attach the cell to the extracellular ma-

trix (ECM) and also work as adhesion molecules for the cell-cell attachments so they were chosen to be studied with integrin  $\beta$ -1 antibody. (Alberts *et al*, 2002)

A tyrosine phosphorylation is one of the most important signaling events occurring at a focal adhesion. The focal adhesions are composed of scaffolding molecules, proteases, lipases, GTPases and enzymes such as kinase phosphatases and there are several types of focal adhesions divided by their composition and subcellular location. They are connected to the actin cytoskeleton through proteoglycans and integrins. A focal adhesion kinase (FAK), a 125 kDa tyrosine kinase and a Src are the two major kinases found in the focal adhesions. Proteins such as FAK, vinculin and paxillin are recruited to the focal adhesion preceding to the tyrosine phosphorylation. FAK activation has shown to cause the phosphorylation of a several key tyrosine residues. FAK phosphorylation in ECM is connected to various signaling pathways that control cell migration and invasion, as well as cell proliferation and survival. Antibodies recognizing FAK were used to study the phosphorylation status of the FAK. (Wozniak *et al*, 2004)

4G10 and KAM-TK150 both bind to the phosphorylated tyrosine residues. 4G10 was used since it recognizes the total phosphorylation of the tyrosine residues while KAM-TK150 recognizes the different epitopes of tyrosine phosphorylated residues.

### 3 EXPERIMENTAL PROCEDURES

HUVECs were cultured and then treated at passage 3-4 either under normoxic or hypoxic conditions for 4 h and 24 h. Some cells were also re-oxygenated for 1 h after the hypoxic treatment.

All the pipettes used in these experiments were annually calibrated by the Finnish national institute for health and welfare (THL). Moreover, most of the solutions and mediums were provided by THL.

#### 3.1 Culturing of HUVEC

Cells were cultured in complete medium containing RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% L-glutamin medium in fibronectin coated bottles. Confluent cells were washed with 4 ml of a phosphate buffered saline (PBS) few times and 1 ml of detachment solution Trypsin-EDTA (HBSS Ca, Mg free, 2% HEPES, 1.5 mM NaHCO<sub>3</sub>, 0.05% trypsin and 0.5 mM EDTA) was added. As a proteolytic enzyme, trypsin digests proteins in the extracellular matrix. With EDTA also cell-to-cell and cell-to-matrix adhesions are removed by chelating the Ca<sup>2+</sup>-ion. Cells were incubated in detachment solution for 5 min. The detachment solution was removed by washing with 1 ml of complete medium and the cells were centrifuged for 1.5 min at 447 x G. Supernatant was discarded and the remaining pellet was re-suspended to 1 ml of EBM2+antibiotics solution (Endothelial basal medium containing antibiotics). Cells were then counted to obtain the optimal cell density. 10 µl of cell suspension was mixed with 90 µl Trypan blue solution and vortexed. 20 µl of this mix was pipetted to the cell counting chamber. The cell counting was performed with an automatic cell counter (Nexcelom Bioscience, Cellometer™ Auto T4). The optimal volume of the cell suspension was calculated based on the cell density counting and 1/3 of the cells were seeded again for further culturing at +37 °C.

### 3.2 Enzymatic assays

Ecto-enzyme activities were measured from the cells after culture under a 4-hour and a 24-hour hypoxia as well as under normoxia and a 1-hour re-oxygenation after a 4-hour and a 24-hour hypoxia.

10 000 cells per each well were applied to clear 96-well plates containing 250  $\mu$ l of D-MEM-0.2 % BSA medium for 24-48 h. The wells were aspirated before the experiment and 60  $\mu$ l of fresh D-MEM-BSA was added. To avoid re-oxygenation shock during medium change, the medium was also equilibrated under hypoxic conditions for 4 h. Incubation times and substrate concentrations were selected so that the amount of radiolabelled metabolites did not exceed 10-15 % of the initially used substrate concentrations.

To measure NTPDase activity, cells were incubated for 20-25 min with 500  $\mu$ M [2,8- $^3$ H]ATP (specific activity 19.0 Ci/mmol, PerkinElmer, USA). 5'-NT activity was measured by incubating the cells for 45 min with 300  $\mu$ M AMP (Sigma) with the tracer [2- $^3$ H]AMP (specific activity 19.7 Ci/mmol, Amersham, GE Healthcare, UK). Adenylate kinase activity was determined by incubating the cells with 500  $\mu$ M [ $^3$ H]AMP for 45 min as respective phosphorous acceptor and 700  $\mu$ M of  $\gamma$ -phosphate donating ATP. In all measurements, 20  $\mu$ l of the substrate was added to start the reaction and incubations were performed in 37 °C waterbath. Also 4 mM of  $\beta$ -glycerophosphate (Sigma) was added to all reaction mixtures to prevent the undesirable hydrolysis of  $^3$ H-nucleotides by non-specific phosphatases.

Reactions were stopped by pipetting 8  $\mu$ l aliquots on TLC plates (0.2 mm Macherey-Nagel Alugram Sil G/UV<sub>254</sub>) to separate  $^3$ H-labelled substrates and their metabolites. 8  $\mu$ l of standard solutions (1 mM ATP, ADP, AMP, adenosine, inosine and hypoxanthine) were pipetted on TLC plates before applying the samples. Standard solutions made the desired bands visible under UV-light so appropriate areas could be scraped off from the silica (see Figure 5). To separate the fractions, TLC plates were run in a glass chamber filled with the eluant. The glass chamber was equilibrated before running the TLC and the eluant was a

mixture of 1-Butanol (Sigma), Iso-amyl alcohol (Sigma), Diethylene glycol monoethyl ether (Sigma), Ammonia solution 32 % (Merck) and Milli-Q-aqua. (9:6:18:9:15). TLC plates were run for ~2 h followed by visualization under UV-light. Bands were marked and silica was scraped off from the marked area with a scalpel to Maxivials (Perkin Elmer).

Samples were extracted into 1 ml of 0.1 M HCl for 1 h at room temperature (RT). 4 ml of HiSafe 3 Optiphase (Perkin Elmer Inc.) was added to vials and let to stay still for one more hour at RT. The vials were placed in a liquid scintillation counter ( $\beta$ -counter, Wallac 1409) and counted twice. Results were calculated from the raw data according to Formula 1. and the enzyme activities were presented as percentual change compared with the enzyme activities in normoxic conditions.

### 3.3 ATP and ADP measurements

Extracellular ATP and ADP concentrations in bathing medium were measured from HUVECs cultured under hypoxic conditions in RPMI-1 % BSA for 4 h and 24 h. Also the effects of 1-hour re-oxygenation on ATP and ADP levels were examined. ATP and ADP measurements were done according to ATPLite™ Kit (Luminescence ATP Detection Assay system, Perkin Elmer) in white 96-well plates. Luminescence was measured with TECAN Infinite series M200 microplate reader (Salzburg, Austria). Nucleotide concentrations were calculated with the calibration curve (Figure 7.) and the results were given in nmol/L.

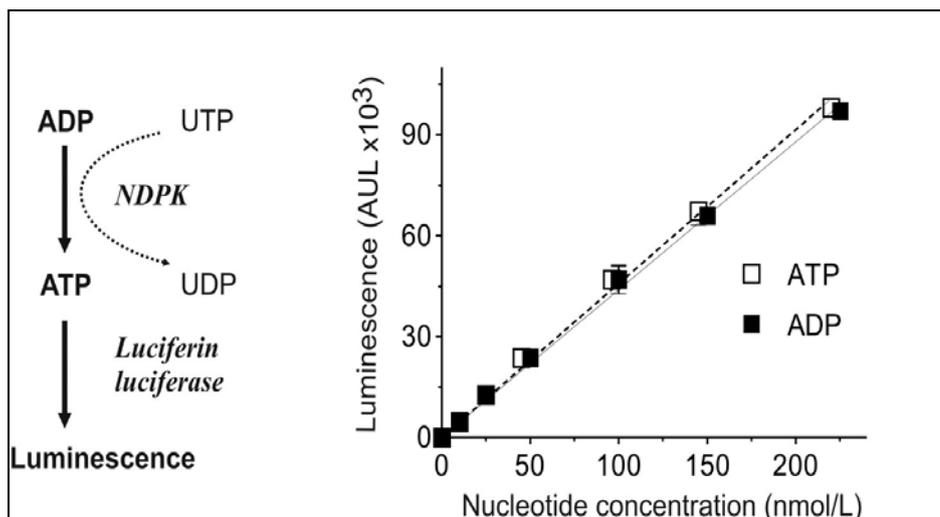


Figure 7. ATP and ADP calibration curve.

### 3.4 Protein measurement

Protein measurements were done according to Pierce BCA Protein Assay Kit. Both, sample analytes and standard solutions made of BSA (ImmunO, Bovine albumin fraction V, pH 7.0, MP Biomedicals Inc.), were diluted to lysis-buffer (Aprotinin 1:100, 10 mM orthovanadate 1:100, 100 mM PMSF 1:1000, 10X Lysis Buffer (20 % saponin- 0.2 % TritonX-100 in PBS), H<sub>2</sub>O). Lysis-buffer was also used as a blank. The protein calibration curve was done with five different concentrations (mg/mL): 0.075, 0.15 , 0.25 , 0.5 and 1.0. Reagents were mixed and 200  $\mu$ L reaction mixture was pipetted to all wells. Samples and standard solution were added in 20  $\mu$ l volume, vortexed and incubated for 30 min in RT. Absorbances were measured with TECAN Infinite M200 microplate reader (Salzburg, Austria) at a wavelength of 562 nm and the results were calculated with the calibration curve (Figure 8).

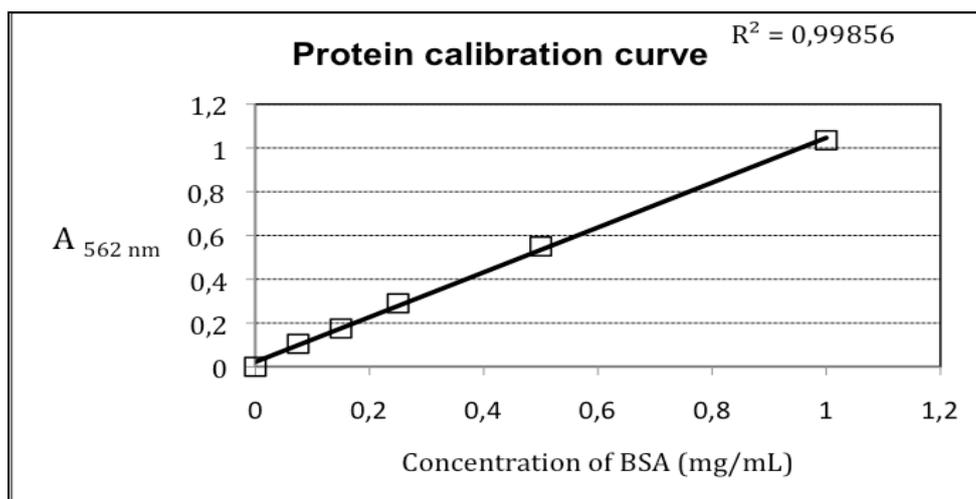


Figure 8. Protein calibration curve.

### 3.5 Immunofluorescence staining of the HUVEC

HUVECs were cultured also for the immunofluorescence analysis. Cells (HUVEC 1) were plated into two 24-well plates containing fibronectin coated cover slips. Cells were let to attach for 24 h and then eight wells from each plate were washed with RPMI and RPMI-1% BSA was added. Cells in six wells remained containing serum. Control plate was cultured in a normal incubator at 37 °C. Other plate instead was cultured in a hypoxic chamber for ~24 h. After hypoxic treatment, cells were washed with PBS and fixed with 4 % paraformaldehyde for 5 min. Paraformaldehyde was washed away with PBS and cells were then permeabilized with 0.2 % TritonX-100 for 3 min and washed with PBS. The stock solutions of the primary antibodies recognizing phosphotyrosine kinase residues 4G10 (Millipore, Massachusetts), KAM-TK150 (Stressgen, Canada), as well as anti- $\beta$ -tubulin (Sigma, USA), Vimentin (Kind gift from Prof. John Eriksson, Åbo Akademi University, Turku), FAK (BD Transduction Laboratories), pFAK (BD Transduction Laboratories), 2C8 (CD31) and integrin  $\beta$ 1 were diluted into the blocking buffer (PBS- 2% BSA- 0.2 % Saponin). Primary antibodies were added to the cells at concentrations of 0.25-1  $\mu$ g/mL and incubated for 30 min at RT.

After incubation with the primary antibodies, wells were first washed with PBS and then several times with the washing buffer (0.2 % BSA- 0.1 % Saponin-PBS) for ~15 min. Appropriate Alexa fluor® 488- or 546- conjugated anti-mouse and anti-chicken (in case of the Vimentin study) secondary antibodies were diluted ~1:100 in the blocking buffer and added to the plates at 150 µL/well. For the concurrent visualization of actin filaments, cells were additionally incubated with an Alexa fluor® Phalloidin 488 and an Alexa fluor® 546 Phalloidin (Invitrogen). The cells were then incubated with a secondary antibody for 20 min at RT. Plates were covered with a foil during the incubation to avoid the fading of the stainings. The wells were washed two times with a washing buffer and once with a PBS. Cells were left in PBS.

Cover slips were now removed from the wells and washed with a distilled water. Vectashield clear mounting medium H-1000 (Vector Laboratories Inc.) was applied to object glasses and cover slips were mounted on the object glasses. Samples were visualized under the fluorescence microscope Olympus BX60 (Japan).

### 3.6 SDS-PAGE and Western Blot

Two sets of HUVECs obtained from different donors were cultured and isolated and both sets of cells were analysed with Western Blot several times using different antibodies.

In the first experiment 100 000 cells were plated to three different six-well plates. One plate was kept under normoxic conditions (control) and the other two were used for 4- and 24-hour hypoxic treatments, respectively.

In the second experiment, there were two different HUVECs. Each plate contained wells with two different cell lines for each experimental condition. Again one plate was for control, one for 4 h hypoxia and one for 24 h hypoxia.

After seeding, the cells were allowed to attach and grow in the plates for two days in a conventional incubator at + 37 °C. Then, the complete medium was replaced in two wells by 2 mL of RPMI-1640/BSA 1 %, while cells in the third well remained in a serum containing medium. Cells were incubated in a hypoxic chamber for 4 h and 24 h. Control cells were cultured in a normal incubator.

The plates were removed from the hypoxic chamber and two wells from each plate were washed with 1 mL of PBS and 350 µL of lysis buffer was added for cell lysis. The cells were lysed for approximately 30 min in a conventional incubator at +37 °C and removed from the wells by scraping. Likewise, after incubation in a conventional incubator for 60 min, the remaining well with “hypoxic and re-oxygenated” HUVEC was subjected to the same procedure of cell lysis and scraping. All lysates were centrifuged for 10 minutes at 7 °C and 13 000 x G. Then, ~300 µL of lysate supernatant was mixed with 5x Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10 % glycerol, 0.01 % Bromphenol Blue, Aqua) containing 10 % mercaptoethanol, heated in 95 °C for 5 min and stored in -20 °C.

10 % acrylamide gels (running gel: 10 % acrylamide, 1.5 M Tris pH 8.8, 10 % SDS, 10 % ammonium persulphate, 0.04 % TEMED and Aqua. Stacking gel: 5.1 % acrylamide and 0.01 % TEMED) were used in all analyses. Frozen lysate samples were thawed out and heated quickly before loading. Samples were loaded into the gel with Rainbow Full range™ molecular weight marker (GE Healthcare, Amersham™). Samples were separated in the gel running at ~100-140 V for 1.5-2 hours. After separation, proteins were transferred from the gel to the nitrocellulose membrane (GE Healthcare; Hybond ECL) by blotting with ~120 V at +4 °C. After proteins had been transferred to the nitrocellulose membrane, the membranes were washed with MilliQ-water and incubated ~15 min in a washing buffer consisting of Tris buffered saline (TBS) and 0.2 % Tween 20. Primary antibodies were diluted 1:1000 in blocking buffer (2 % BSA in TBS-Tween20 0.2 %). Primary antibodies used were 4G10 , KAM-TK150 , p-FAK, FAK, actin and anti-β-tubulin. Primary antibodies were incubated over night with rotation at + 4 °C. After incubation with the primary antibodies, the membranes

were washed with a washing buffer three times for ~15 min at RT. HRP-conjugated secondary antibody (Rabbit anti-mouse HRP, Dako code no:P0260) was diluted to a blocking buffer with the ratio of 1:2500. Membranes were incubated with the secondary antibody for 1 h at RT in rotation. Membranes were then washed three times for ~15 min with washing buffer and once for ~30 min with TBS. Membranes were developed with ECL Detection Kit (Amersham) according to manufacturer`s instructions. Image of the separated proteins was developed into a film (Amersham hyperfilm). Instruction are available online at: [http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/4DE67EABFB9A9D25C1257628001CDC12/\\$file/28955347AD.pdf](http://www.gelifesciences.com/aptrix/upp00919.nsf/Content/4DE67EABFB9A9D25C1257628001CDC12/$file/28955347AD.pdf)

## 4 RESULTS AND DISCUSSION

### 4.1 Effects of hypoxia on endothelial purine converting ectoenzymes and extracellular nucleotide levels

Enzyme activities were determined during acute hypoxia (1 %; O<sub>2</sub>) and re-oxygenation after hypoxia. Responses were measured after 4-, 24- as well as after 1-hour re-oxygenation in a conventional incubator after 4-hour hypoxia.

As shown in Figure 9 A, endothelial ATPase activities remained unaltered after 4-24 hour hypoxia whereas 1 hour re-oxygenation after 4-hour and 24-hour hypoxia reduced ATPase activities significantly.

Both 4-hour and 24-hour hypoxia increased 5'-nucleotidase activities (Figure 9 B) 20 % and 15 %, respectively. 1-hour re-oxygenation in a conventional incubator increased 5'-NT activities even to 25 % and 20 % compared with the control level.

Moreover, according to the results depicted in Figure 9 C, adenylate kinase activity was increased after 4-hour hypoxia but the activity was reduced back to the control level after 1-hour re-oxygenation. By contrast, 24-hour hypoxic exposure of endothelial cells with or without subsequent re-oxygenation did not affect on adenylate kinase activity, as compared with control HUVEC maintained at normoxic conditions.

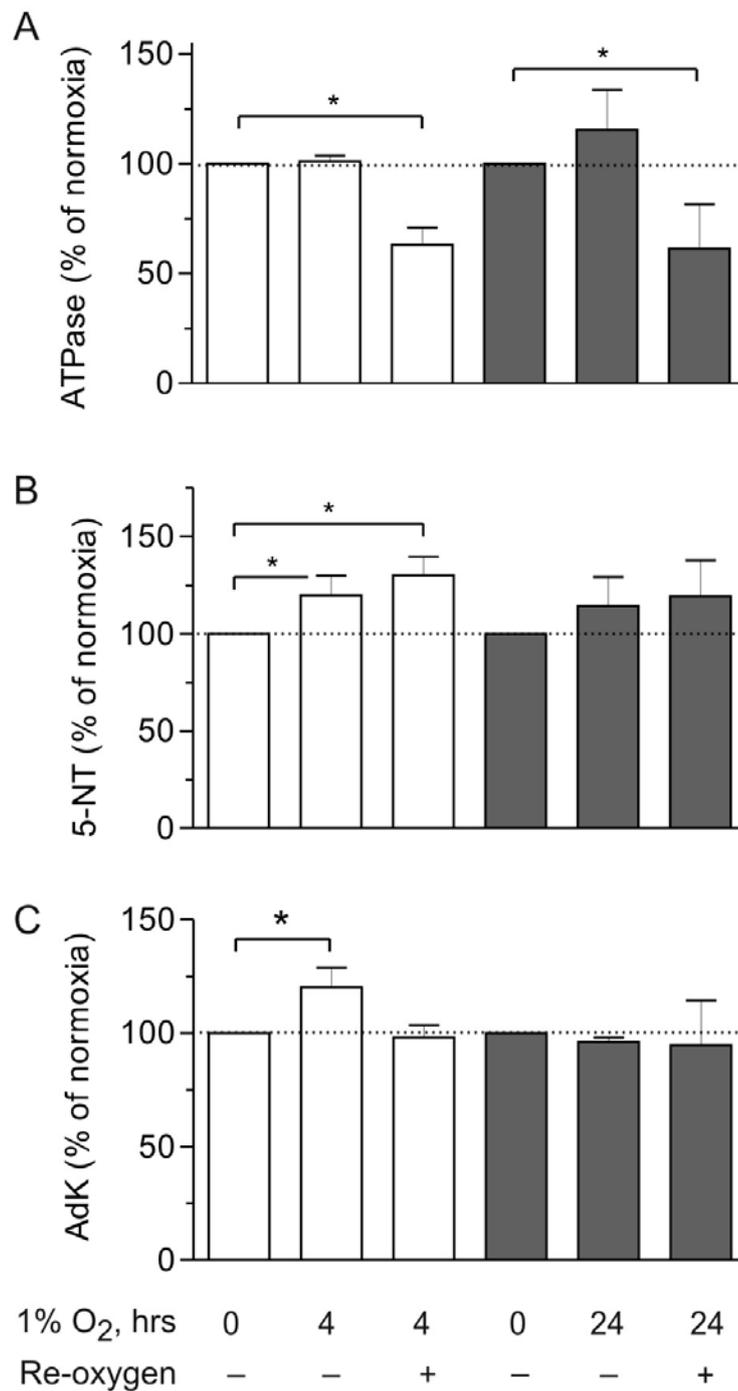


Figure 9. Effects of acute hypoxia and re-oxygenation on the pattern of nucleotide metabolism in cultured HUVEC. Results are presented as percentual change in enzyme activities under hypoxic conditions compared with enzyme activities in normoxic conditions. Statistical significance was determined between control and hypoxic HUVEC (\*  $p < 0.05$ )  $n=5$ .

To study the effects of acute hypoxia on extracellular nucleotide levels, ATP and ADP levels were measured in the bathing medium. The measured ATP and ADP levels were approximately 5 nmol/L and 3 nmol/L in serum free samples respectively, and these extracellular nucleotide concentrations were not elevated under hypoxic conditions.

The results show that hypoxic conditions did not increase the secretion of ATP or ADP to the extracellular milieu, at least on a constitutive rate. It should be noted that the nucleotide concentrations were measured only from serum free samples since fetal calf serum causes nucleotide scavenging by the enzymes and there will not be any extracellular ATP or ADP.

Previous studies with different types of endothelial and other cells revealed contradictory findings on the expression levels and activities of major ecto-nucleotidases during hypoxic exposure. For instance, dramatic increases in both NTPDase and ecto-5'-nucleotidase activities were observed in post-hypoxic endothelial and epithelial cells (Eltzschig *et al*, 2003, Li *et al*, 2006), as well as in murine models of acute hypoxia, which were additionally accompanied by the marked vascular leakage in the lungs (Eltzschig *et al*, 2006).

On the other hand, acute hypoxia (<24 hours) was shown to have no effect on endothelial ecto-nucleotidase activities but triggered substantial decreases in cell-surface ecto-ATPase and ecto-5'-nucleotidase activities in fibroblasts (Gerasimovskaya *et al*, 2002, Robson *et al*, 1997).

Our data support the latter findings on minor hypoxia-induced changes in endothelial nucleotidase activities and further demonstrate that a subsequent post-hypoxic re-oxygenation shock may be even more crucial for modulating enzymatic activities. Moreover, data on increased adenylate kinase activity in acutely hypoxic HUVEC suggest that counteracting ATP-regenerating pathway may be concurrently upregulated during hypoxic exposure.

Extracellular ATP has shown to be pro-angiogenic factor for endothelial cells and this could explain why high levels of ATP are maintained during hypoxic conditions. (Gerasimovskaya *et al*, 2007).

Since ATP and ADP were not shown to be released in nucleotide measurements, nucleotides might be provided *in vivo* conditions by surrounding cells such as leucocytes and SMCs and pericytes.

#### 4.2 Effects of acute hypoxia on cytoskeleton components and signaling pathways in endothelial cells

4G10 is an antibody which binds to the phosphorylated tyrosine residues. The total phosphorylation was decreased in both, complete serum containing medium and RPMI- 1% BSA after 24-hour hypoxia as seen in Figure 10.

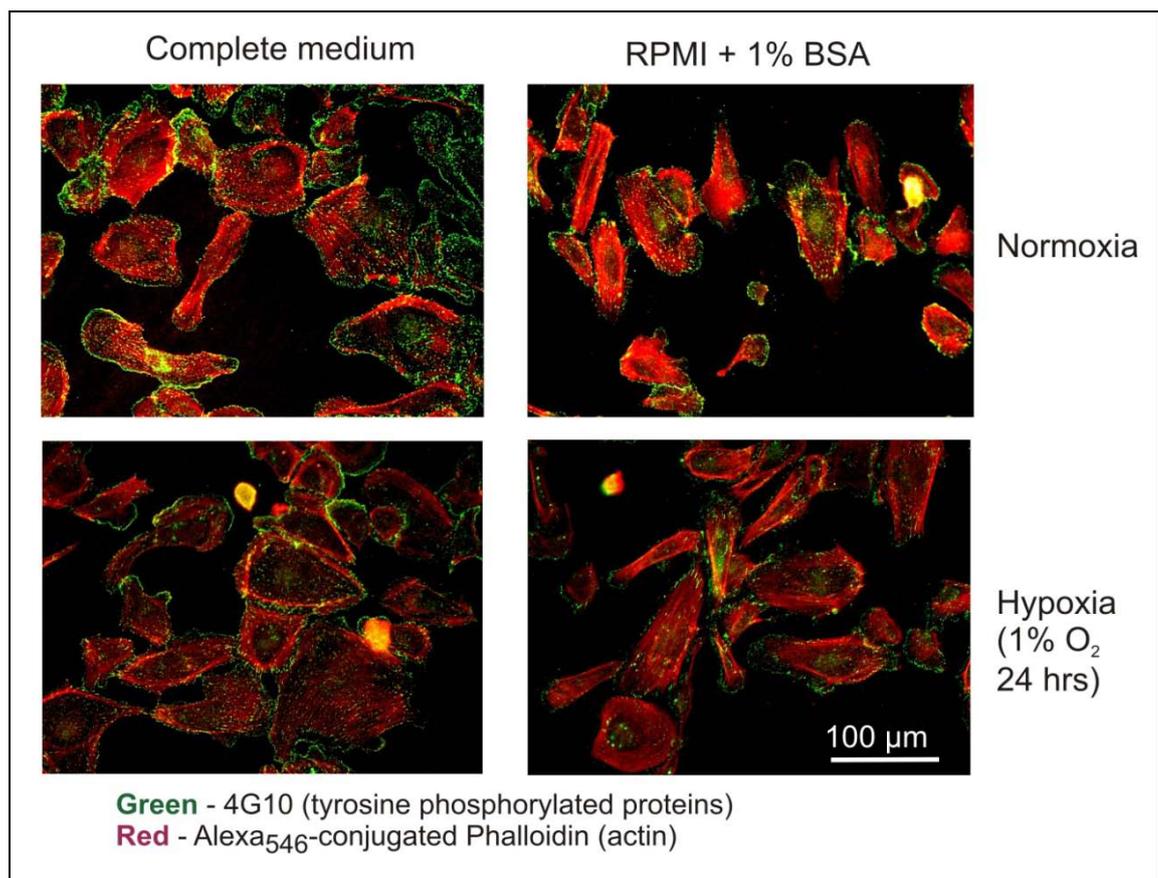


Figure 10. HUVEC immunostainings with 4G10 in complete medium and in RPMI-1 % BSA at hypoxia and normoxia. Green colour shows labelled actin filaments and red colour shows the tyrosine phosphorylated proteins.

Hypoxia seems to decrease the activation status of the endothelial cells. The activation status of the endothelial cells is higher in complete medium which shows the importance of the “starvation” of the cells.

Likewise, similar staining pattern and decreased phosphorylation were observed using another antibody KAM-TK150, which recognizes different epitopes of tyrosine phosphorylated residues than 4G10 (Figure 11.).

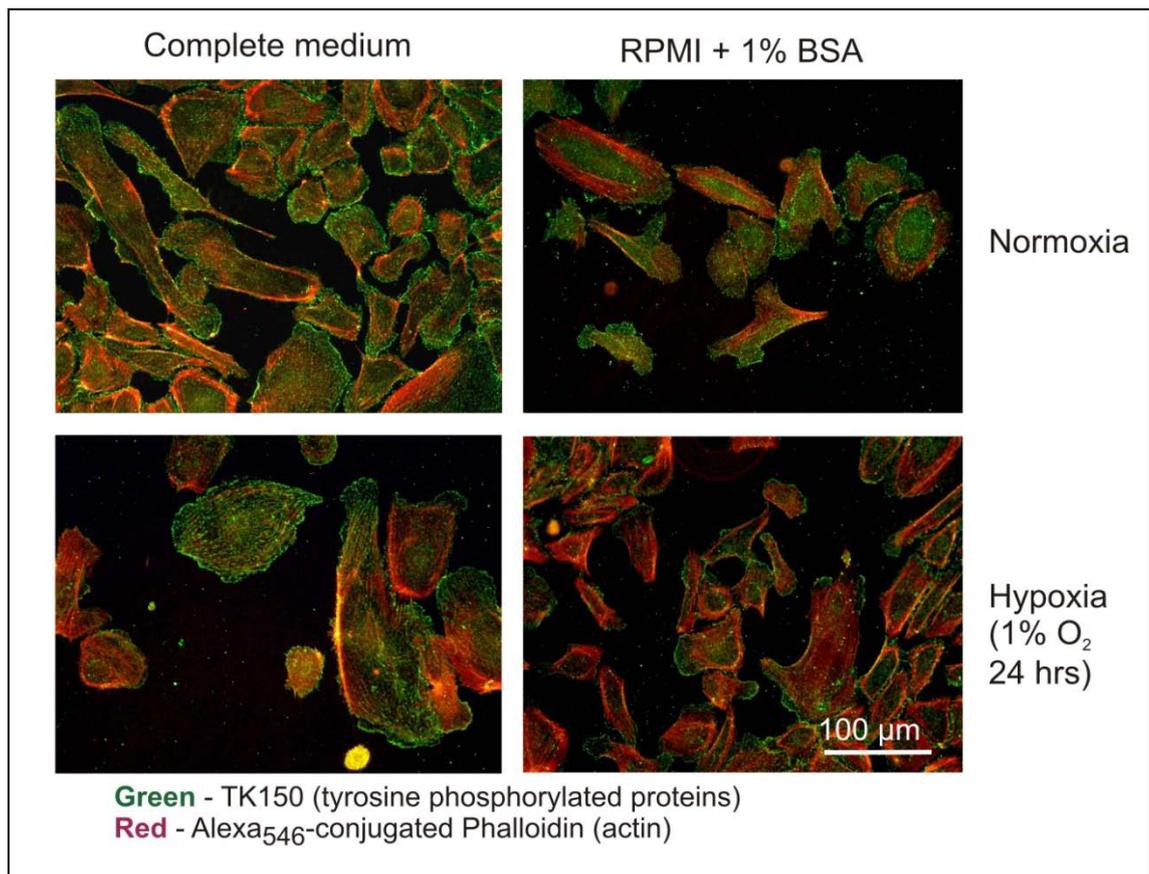


Figure 11. HUVEC immunostainings with TK150 in complete medium and in RPMI-1 % BSA under hypoxic and normoxic conditions. Cells were stained with primary antibody TK150. Pictures show that phosphorylation has decreased in both complete medium and RPMI-1 % BSA after 24-hour hypoxia.

Since tyrosine phosphorylation is related to the tyrosine kinase receptor activation which then activates several important extracellular signaling pathways, decreased phosphorylation status reflects the metabolic state of the cell. Hypoxia seems to “shift” cells to less active state.

At the expression levels of the major cytoskeleton components, tubulin (left panel) and vimentin (right panel), as well as actin are shown on subsequent figures (Figure 12).

It should be emphasized that subsequent staining experiments were performed using "quiescent" HUVEC growing in serum-free medium and exposed to normoxia or hypoxia. No significant difference between normoxia and 24-hour hypoxia was observed with neither tubulin nor vimentin.

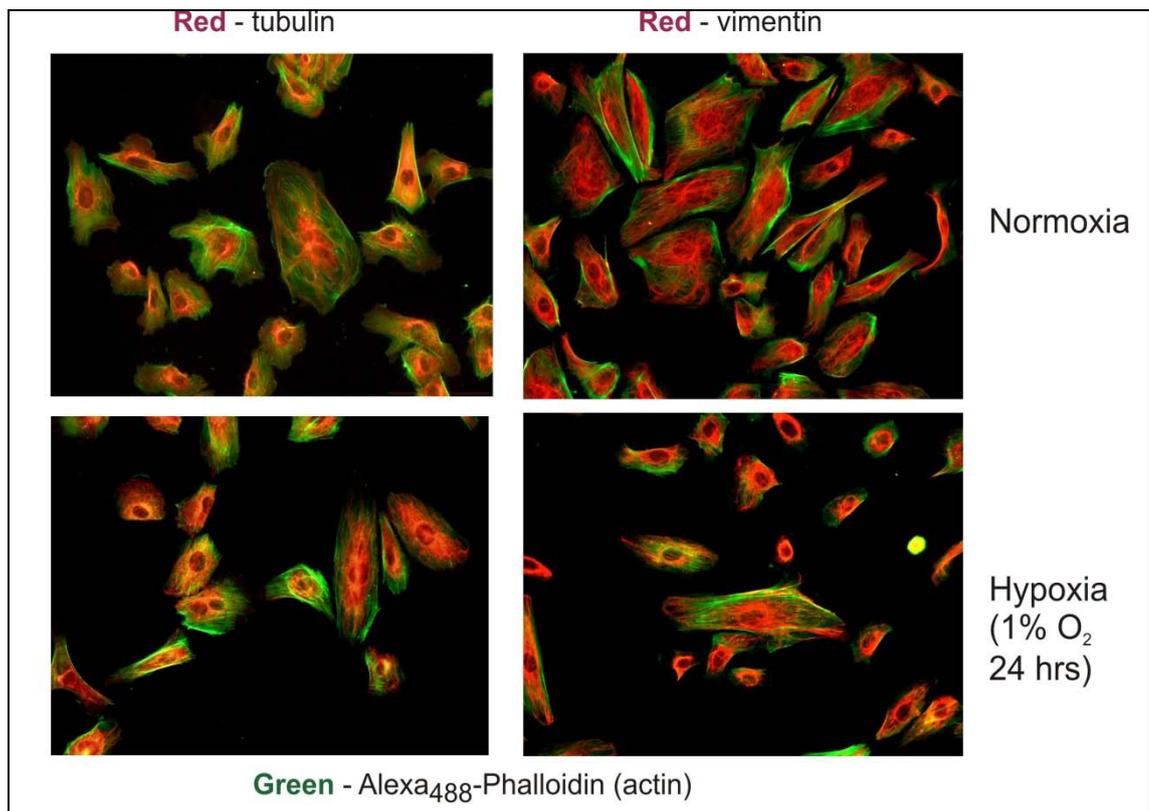


Figure 12. Cytoskeleton elements tubulin and vimentin in HUVECs at normoxia and after 24-hour hypoxia (1%; O<sub>2</sub>). Tubulin and vimentin structures are shown as red and actin filaments are shown as green.

Hypoxia does not affect to the cell shape and viability of the endothelial cells. According to Gerasimovskaya *et al*, 2007, ATP induces rearrangement of the cells in areas of active angiogenesis to a tube-like network in vasa vasorum endothelial cells. Since HUVECs did not release ATP, this phenomenon was not observed in our studies.

Given the important role of focal adhesion kinases in the regulation of cell adhesion, migration and proliferation in a variety of cell types, including vascular endothelial cells, the aim was to determine whether the phosphorylation status of this molecule is somehow changed during HUVEC exposure to hypoxia. As can be seen in Figure 13. HUVEC staining with antibodies against total pool (left panels) and phosphorylated (right panel) FAK revealed clear green punctuate stainings located beneath plasma membranes in the control normoxic cells, which were substantially "faded" after HUVEC exposure to hypoxia

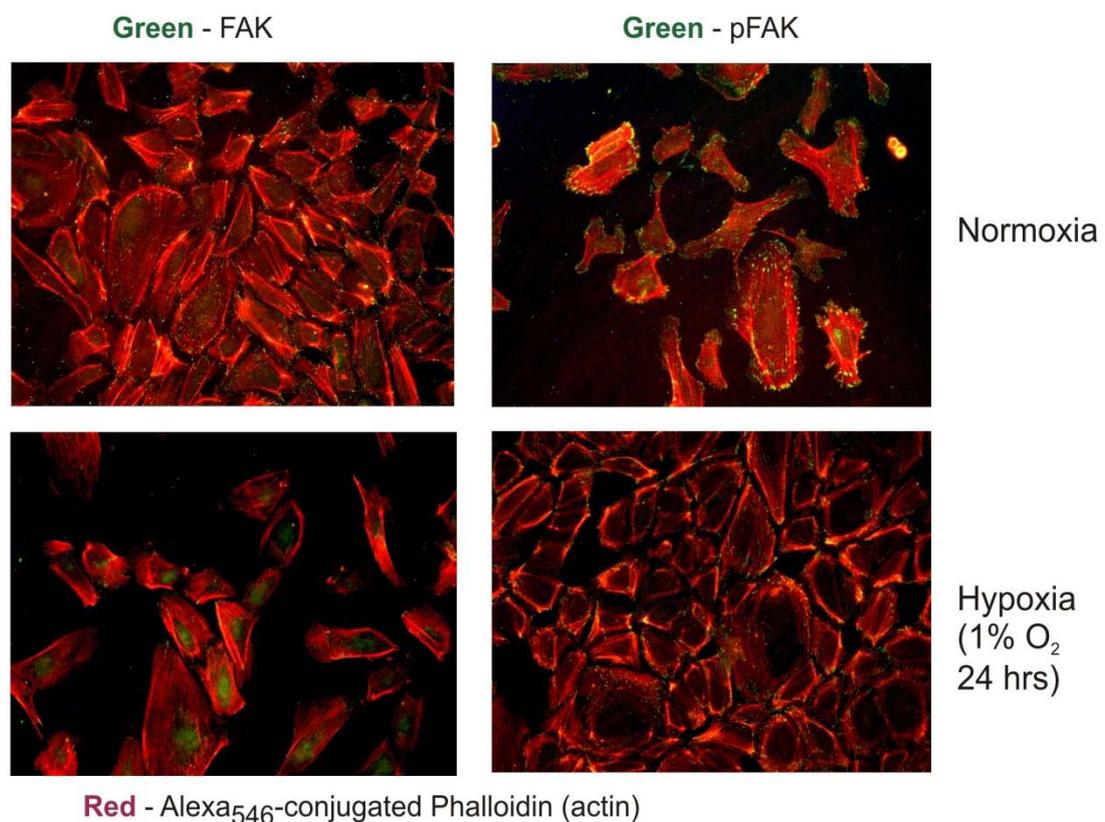


Figure 13. HUVECs labelled with anti-FAK and anti-pFAK antibodies at normoxia and after 24-hour hypoxia (1%; O<sub>2</sub>).

The effects of hypoxia in the FAK expression on endothelial cells need to be further studied. It would be interesting to see whether mRNA levels would be altered during acute hypoxia.

Staining of the HUVEC with endothelial cell marker CD31, also known as PECAM-1 confirmed the predominant localization of this molecule in cell junctions and further demonstrated marked up-regulation of CD31 expression after 24-hour hypoxia. Upregulation might be related to the increased adhesion capability.

At the right hand side panel in Figure 14. HUVEC are labelled with  $\beta 1$ . Integrins are shown in a picture as red. A great difference after 24-hour hypoxia is seen in integrin amount and distribution. Reason for this phenomenon still remains unknown.

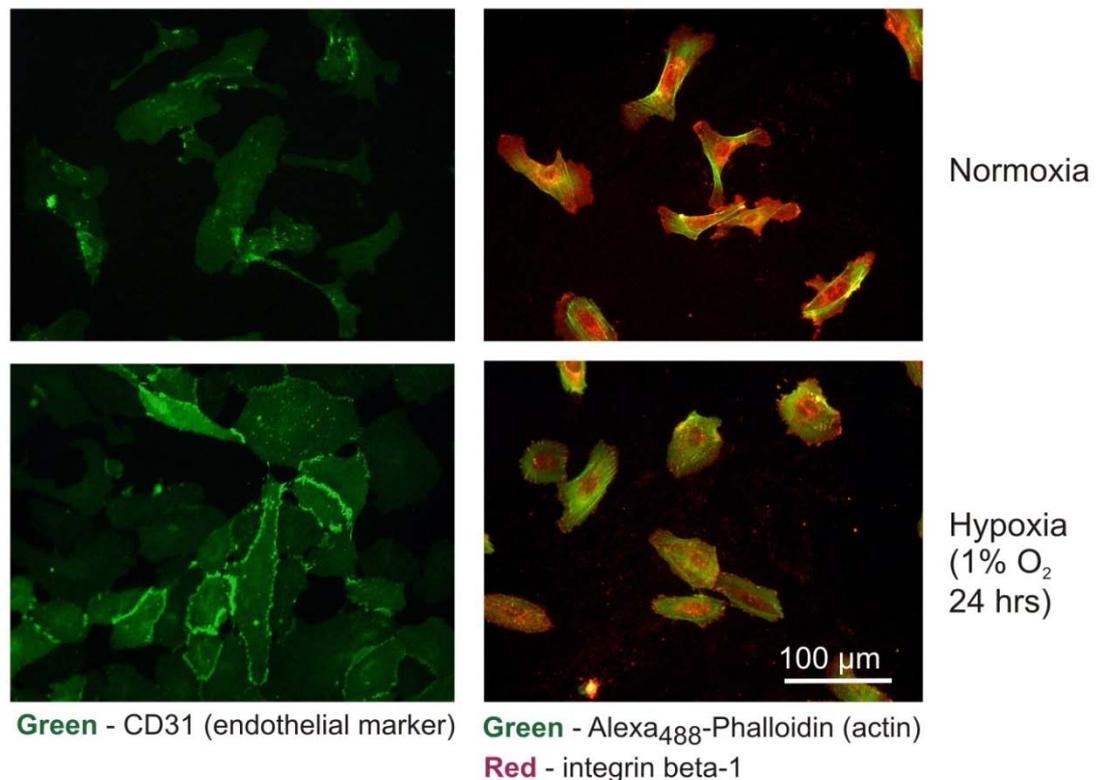


Figure 14. HUVECs immunofluorescence with CD31 and integrin beta-1 at normoxia and after 24-hour hypoxia (1 %; O<sub>2</sub>). At left side panel cells stained with CD31 and at right side panel cells stained with  $\beta 1$ .

Studies with 4G10 revealed that short-term "starvation" of HUVEC in the absence of serum was accompanied by diminished amount of tyrosine-

phosphorylated proteins, as compared with HUVEC growing in complete media (Figure 15.).

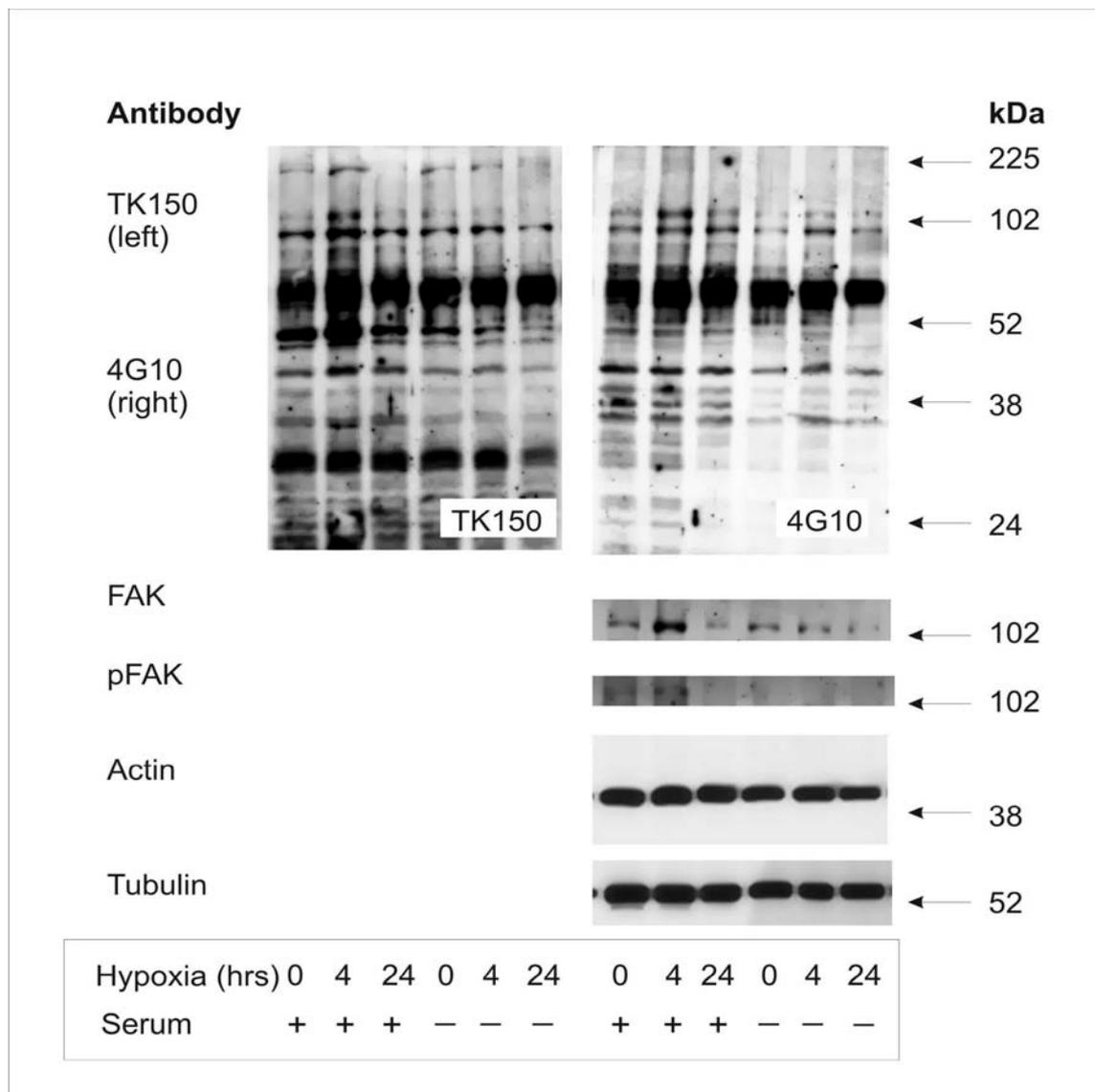


Figure 15. Images of Western Blots stained with FAK, pFAK, Actin, Tubulin.

After 4-hour hypoxia phosphorylation of FAK was upregulated. Interestingly, acute hypoxia affected to the pattern of protein phosphorylation and particularly manifested in decreased amounts of both total and phosphorylated FAK after 24-hour hypoxic exposure as seen in Figure 16.

Protein levels vary slightly between the wells, even though the protein levels were adjusted to be as similar as possible. This complicates drawing conclusions. However, some major differences were detected.

### 4.3 Summary

According to the results, acute hypoxia seems to have only minor effects on human umbilical vein endothelial cells in terms of ecto-enzyme activation levels and focal adhesion kinase. Contrary to the previously proposed concepts postulating that acute hypoxia might trigger massive release of extracellular nucleotides accompanied by up-regulation of endothelial ecto-nucleotidase activities, our data revealed relatively moderate hypoxia induced changes in NTPDase and ecto-5'-nucleotidase activities without any increase in basal ATP and ADP levels.

This thesis was part of more extensive project and research on the effects of acute hypoxia will be continued in future.

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