

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

THE ROLE OF PROTEIN PHOSPHATASE 2A IN THE LUNG  
ENDOTHELIAL BARRIER REGULATION

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# **The role of protein phosphatase 2A in the lung endothelial barrier regulation**

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The examination takes place at the Department of Physiology, Medical and Health Sciences Center, University of Debrecen, December 16<sup>th</sup>, 11:00 h

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December 16<sup>th</sup> 2013, 13:00 h

## **1. Introduction**

### **1.1. The lung vascular barrier**

#### **1.1.1. The heterogeneity of endothelial cells**

The vascular endothelium serves as a semi-permeable barrier lining in the vessel walls. It dynamically regulates the liquid and macromolecule transport between the blood and the interstitial space. The vasculature is lined by heterogeneous population of endothelial cells. Their heterogeneity is derived from the origin of endothelial cells in the vascular tree. Pulmonary artery endothelial cells (macrovascular ECs) play important physiological and pathophysiological role in the human body. For example, they participate in blood homeostasis, blood-tissue exchange regulation under various conditions. They share similarities in cell characteristics and in physiological properties with microvascular ECs. However, *in vivo* models of clinical pulmonary edema suggest that most fluid filtration occurs in the microcirculation. Mostly due to technical limitations in the isolation and culturing of MVECs, the majority of *in vitro* data on mechanisms controlling pulmonary EC barrier properties has been obtained from macrovascular ECs.

#### **1.1.2. Clinical and physiological importance of endothelial barrier in the lung**

Direct or indirect injuries of the lung caused by inflammatory or toxic mediators can lead to pathological syndromes of severe pneumonia, acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS). The acute phase of acute ALI and ARDS is characterized by a massive and rapid flood of protein rich edema fluid into the alveolar spaces as a consequence of increased endothelial permeability. It is widely accepted that EC barrier dysfunction, a prominent feature of these clinical syndromes is tightly linked to agonist-induced cytoskeletal remodeling, which leads to the disruption of cell-cell contacts, paracellular gap formation and EC barrier compromise.

### **1.2. Mechanisms regulating endothelial permeability**

#### **1.2.1. Endothelial permeability pathways**

Permeability may be separated into basal and induced permeability. The former occurs at the level of microvascular ECs, while the latter is often associated with inflammation, involving macrovascular ECs. A variety of physical, inflammatory and bioactive stimuli alter the EC barrier leading to gap formation, increasing vessel permeability and compromising organ function. Permeability across endothelial cell monolayers can involve transcellular, paracellular or the combination of both pathways. However, the majority of trafficking occurs

through the paracellular pathway. These pathways are regulated by the network of cytoskeletal and cell-junction elements which will protect endothelial barrier integrity.

### **1.2.2. Permeability increasing agonists**

During inflammation the endothelium is activated by mechanical alterations, by agonist stimuli such as thrombin, histamine, TNF $\alpha$  or reactive oxygen species. Thrombin is a serine protease generated by injured endothelial cells by the cleavage of circulating prothrombin. Thrombin binds to G-protein-coupled protease activated receptors (PAR-1, PAR-2, and PAR-3) and is implicated in several cellular effects. It is an important factor in the development of ALI and ARDS.

### **1.2.3. Regulation of endothelial paracellular gap formation**

Endothelial barrier integrity is maintained by the precisely regulated balance between actin-myosin contractile forces and adhesive cell-cell, cell-matrix tethering forces. Both competing forces are tightly linked to the cytoskeleton comprising of actin microfilaments, microtubules and intermediate filaments. Regulation of EC barrier function is critically dependent upon activities of protein kinases and protein phosphatases. Specifically, cell junction proteins, cytoskeletal, or cytoskeleton-associated proteins are regulated by the precise mechanism of phosphorylation/dephosphorylation events. Under basal conditions cortical actin ring can be observed where endothelial cells can maintain tight connections with each other and with the underlying matrix. Due to the effect of barrier compromising agents, for example thrombin, contractile forces dominate. Thrombin increases intracellular Ca<sup>2+</sup>, which will activate the Ca<sup>2+</sup>/calmodulin dependent myosin light chain kinase (MLCK) that will phosphorylate MLC leading to cell contraction. Thrombin also activates the small GTPase Rho signaling pathway which results in barrier dysfunction.

## **1.3. Cell junctions in the endothelium**

Communicating structures like adherens junctions (AJ) link ECs together and regulate endothelial permeability. Adherens junctions represent the majority of cell junctions comprising the endothelial barrier. Endothelial cells contain vascular endothelial (VE)-cadherin as a transmembrane protein that mediates the binding of adjacent cells. The intracellular tail of VE-cadherin binds p120 catenin,  $\beta$ -catenin or plakoglobin which attach  $\alpha$ -catenin to link the cadherin–catenin complex to the actin cytoskeleton. VE-cadherin function is necessary for the proper assembly of AJs, and for normal endothelial barrier function.

$\beta$ -catenin has a dual role in the cells. First it was identified as a component of AJs in the late '80s. Later genetic and embryogenic studies revealed  $\beta$ -catenin as part of Wnt-signaling

pathway. Plakoglobin is closely related to  $\beta$ -catenin, sharing 80 % sequence identity and could bind the cytoplasmic domains of the classical cadherins. Both  $\beta$ -catenin and plakoglobin were shown to stabilize the linkage between VE-cadherin and the actin cytoskeleton, thus regulating endothelial barrier function

### **1.3.1. Regulation of junctional permeability- reversible phosphorylation in AJs assembly**

The dynamic assembly and disassembly of adherens junctions depends on protein-protein interactions regulated by reversible phosphorylation. Recent studies revealed the possibility of AJ regulation by Ser/Thr phosphorylation. There is a growing body of evidence on the Ser/Thr phosphorylation and the stabilization of cadherin/catenin complex.  $\beta$ -catenin, as part of the Wnt-signaling, directly associates with Ser/Thr kinases: casein kinase I (CKI) and GSK-3 $\beta$ . These kinases induce the phosphorylation of  $\beta$ -catenin on Ser45, Ser33/37 and Thr41, respectively, leading to its ubiquitination and proteosomal degradation. PKA was also shown to phosphorylate  $\beta$ -catenin at Ser552 and Ser675 sites which induce  $\beta$ -catenin transcriptional activity. Despite of the significance of these proteins' phosphorylation the phosphatases which can turn the process reversible are still unknown.

### **1.4. Reversible protein phosphorylation**

Reversible protein phosphorylation has been known to control a wide range of biological functions and activities such as cell cycle, apoptosis, growth, differentiation and it is involved in almost every signal transduction pathway in all living organisms. Reversible phosphorylation of proteins is driven by opposing activities of protein kinases and protein phosphatases. Protein kinases catalyze the transfer of terminal  $\gamma$ -phosphate group of adenosine triphosphate (ATP) to hydroxyl-containing amino acid side chains: serine, threonine and tyrosine, while protein phosphatases facilitate the hydrolysis of phosphate groups from the phospho-Ser-, -Thr- or -Tyr- side chains of proteins.

### **1.5. Classification of protein phosphatases**

Two types of protein phosphatases have been identified, according to their specificity to dephosphorylate amino acid side chains. Ser/Thr protein phosphatases (PP) specifically hydrolyze phospho-Ser/Thr phosphoesters, while protein tyrosine phosphatases (PTP) hydrolyze phospho-Tyr. Tyr-specific phosphatases can be further classified into phospho-Tyr-specific PTP and dual-specificity protein Tyr phosphatases, DS-PTP, which can catalyze dephosphorylation of both Tyr and Ser/Thr residues. The PP family first was classified into type 1 (protein phosphatase 1, PP1) and type 2 (protein phosphatase 2, PP2) based on their substrate specificity and their sensitivity towards heat stable phosphatase inhibitors (inhibitor-1 and inhibitor-2). Type 2 phosphatases were then further divided into three subclasses

according to their metal ion dependency. PP2A does not require metal ion to its function. PP2B turned out to be a  $\text{Ca}^{2+}$  /calmodulin dependent phosphatase, also known as calcineurin. PP2C found to be  $\text{Mg}^{2+}$  dependent, which was later identified by sequence analysis, as a different superfamily: metal ion dependent protein phosphatase, PPM. According to a new classification, Ser/Thr specific phosphatases fall into three families based on their sequence and their catalytic mechanisms. These are the phosphoprotein phosphatases (PPP), metal- ion dependent phosphatases (PPM) and FCP/SCP aspartate phosphatases. Members of PPP family involve PP1, PP2A, PP2B (calcineurin) and novel- type phosphatases, PP4, PP5, PP6, and PP7. PP1 and PP2A are the most abundant phosphatases and they are involved in many cellular functions.

### **1.6. Characterization of protein phosphatase 1**

Protein phosphatase 1 is ubiquitously expressed in eukaryotic organisms, and responsible for numerous cellular processes in the cell, for example, cell division, apoptosis, metabolism, cytoskeletal reorganization. This phosphatase is highly conserved (~70 %) in mammals during evolution. PP1 consists of a catalytic subunit and a regulatory subunit. The catalytic subunit comprises of three isoforms: PP1 $\alpha$ , PP1 $\beta/\delta$ , and PP1 $\gamma$ . PP1 has several interacting protein partners which function as targeting subunits, substrates and/or inhibitors. Up to now more than 100 putative R subunits of PP1 have been identified. These PP1 binding subunits share no common features, except the short conserved PP1c binding motif RVxF/W.

#### **1.6.1. Myosin Phosphatase (MP)**

EC contraction is initiated by the reversible phosphorylation of the 20 kDa myosin regulatory light chain (MLC) at Ser19 and Thr18 amino acid residues, which is intimately linked to F-actin filament reorganization. There are two key enzymes involved in MLC phosphorylation level regulation: MLCK and MP. MP is a type-1 protein phosphatase, composed of a catalytic subunit, PP1 C $\delta$  and two non-catalytic subunits, the 20 kDa small regulatory subunit (M20) and a 110-130 kDa regulatory subunit, called myosin phosphatase targeting subunit 1 (MYPT1). The exact function of M20 is not identified yet. The direct involvement of PP1 in the regulation of endothelial barrier function and gap formation was shown by several research groups.

#### **1.6.2. Regulators of myosin phosphatase**

MP functions are regulated via the activation or the inhibition of the enzyme. The inhibitory events are well characterized. The most important regulation occurs through the phosphorylation of MYPT1. The existence of specific inhibitory proteins such as CPI-17 (PKC potentiated inhibitory protein of 17 kDa) further increased the complexity of MP regulation. For example CPI-17 blocks not only the catalytic subunit of PP1, it is able to inhibit the MP

holoenzyme without dissociating its subunits. This soluble globular protein composed of 147 amino acids was first shown in smooth muscle cells, and later was found in platelets and in brain. CPI-17 is well conserved in mammals. Phosphorylation of CPI-17 at Thr38 by PKC increased its inhibitory potency toward MP ~1000-fold. Previously published studies demonstrated expression of endogenous CPI-17 in human lung microvascular endothelial cells (HLMVEC) and confirmed its role in the regulation of EC cytoskeleton and permeability. Importantly, the activity of CPI-17 is controlled by PP2A that initiates its dephosphorylation suggesting a novel possibility in MP regulation.

## **1.7. Protein Phosphatase 2A**

### **1.7.1. Structural features of PP2A**

PP2A exists in two forms in the cells: the heterodimer core enzyme and the heterotrimer holoenzyme. The core dimer (PP2A<sub>D</sub>) contains a 65 kDa structural subunit (A subunit or PR65) and a 36 kDa catalytic subunit (PP2A C). The core enzyme associates with a third variable regulatory B subunit to assemble into a holoenzyme.

The catalytic C subunit has two distinct isoforms, C $\alpha$  and C $\beta$  sharing 97 % sequence identity. Two different genes ( $\alpha$  and  $\beta$ ) encode the PP2A A subunit. The majority of the enzymes contain PP2A A $\alpha$  isoform and a small fraction stands for PP2A A $\beta$ . These two isoforms share 87 % sequence homology. The regulatory B subunit has four unrelated families encoded by multiple genes, with multiple splice variants creating a huge diversity of regulatory subunits and isoforms. B (PR55, B55) has four isoforms:  $\alpha$ ,  $\beta$ ,  $\gamma$ , or  $\delta$ ; B' is named as PR61, B56, or RTS1 ( $\alpha$ ,  $\beta$ ,  $\gamma$ 1-3 $\delta$  1-3 $\epsilon$  isoforms), B'' (PR48/PR72/PR130) also have several isoforms ( $\alpha$ 1, 2,  $\beta$ ,  $\gamma$ ) and the B''' is also known as PR93/PR110. Biochemical and genetic studies revealed that the regulatory B subunits are responsible for targeting the PP2A<sub>D</sub> to their substrates and for the subcellular localization of the enzyme. A subunit forms an elongated horseshoe - shape fold, which binds the catalytic C and the regulatory B subunits. The crystal structure of the heterotrimer PP2A holoenzyme confirmed that the regulatory B subunit interacts with C subunit. All B subunits were shown to directly interact with the PP2A core enzyme except the B''' family. It was estimated that the combination of the A-B-C subunits can produce more than 75 different holoenzymes, although the precise number of the possible holoenzyme complexes is yet to be determined.

### **1.7.2. Regulation of PP2A**

#### **The catalytic subunit**

The catalytic subunit of PP2A (PP2Ac) is a large conserved domain. Similarly to PP1c, PP2Ac associates with many regulatory subunits which target the enzyme to various substrates and subcellular locations. The catalytic subunit undergoes reversible carboxyl-methylation and demethylation at its C-terminal leucine residue (Leu309) catalyzed by PP2A-methyltransferase (PMT) and PP2A methyltransferase (PME-1), respectively. Reversible carboxy-methylation of PP2A C is a conserved regulatory mechanism in the enzyme function. However, physiological effect of this modification is controversial. Besides the carboxy-methylation, PP2A was also shown to be effected by tyrosine kinases, epidermal growth factor and insulin receptors which have been shown to inactivate PP2A. The activity of PP2A can also be regulated by toxins, such as okadaic acid, microcystine or calyculin A. More recently a phosphate ester antibiotic, fostriecin, also turned out to be a strong and relatively specific inhibitor of PP2A

#### **The structural A subunit**

The PR65 or A subunit is the structural core of the PP2A holoenzyme tightly associated with the catalytic subunit forming binding site for the B regulatory subunit. The scaffolding A subunit structure is entirely composed of 15 repeats of HEAT motif, built from 39 amino acids. HEAT repeats are highly conserved sequences composed of two  $\alpha$ -helices with an interhelical loop. Two alternative genes encode the two isoforms of PP2A A. A $\alpha$  and A $\beta$  share high homology, although due to the unique structure of PR65 $\beta$  it is unable to substitute for PR65 $\alpha$  in knockout mice

#### **The regulatory B subunit**

The variable B subunit family has four different subfamilies that are considered as B, B', B'' and B'''. These proteins share no structural or functional similarities. Furthermore, the number of isoforms and splice variants within each subfamily increases their variability. AB $\alpha$ C holoenzyme is the most abundant and ubiquitous PP2A holoenzyme, and B $\alpha$  (B55) is the most predominant regulatory subunit of PP2A. Several publications mention its involvement in cellular functions, and distinct subcellular localizations. For instance, B $\alpha$  associates with microtubules, neurofilaments, intermediate filaments as well as cytoplasmic, membrane and nuclear proteins. The B family members are highly conserved, B55 contains four to seven degenerate WD40 repeats. They comprise of approximately 40 amino acids that typically end in tryptophan-aspartate (WD) and are thought to be responsible for protein-protein interactions. The B55 $\alpha$  subunit makes extensive contacts with the A scaffolding subunit but little contact with the C catalytic subunit.



### **1.7.3. Biological functions of PP2A**

PP2A has an important role in the control of cell cycle, transformation, growth or apoptosis, therefore it is involved in several signaling pathways. Some literature data suggest that PP2A acts as a tumor suppressor. PP2A exerts inhibitory effects on Wnt/ $\beta$ -catenin signaling that have a significant role in development and tumorigenesis. Multiple kinases involved in  $\beta$ -catenin phosphorylation such as casein kinase I (CKI) and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) leading to the ubiquitination of  $\beta$ -catenin. PP2A B $\alpha$  was shown to directly interact with  $\beta$ -catenin and it regulates  $\beta$ -catenin dephosphorylation in Wnt-signaling in colon carcinoma cell lines.  $\beta$ -catenin also associates with Axin, GSK3- $\beta$  and APC. The latter one was shown to bind PP2A B subunits, PR61 $\alpha$  and PR61 $\delta$  with bacterial two hybrid screening. PP2A can stabilize  $\beta$ -catenin directly. PP2A has been reported to form a complex with E-cadherin and  $\beta$ -catenin at the plasma membrane resulting in the stabilization of both proteins.

### **1.7.4. PP2A and the cytoskeleton**

The endothelial cytoskeleton comprises of actin microfilaments, microtubules (MT) and the intermediate filaments (IF), regulating cell shape and dynamics. Reversible phosphorylation of cytoskeletal or cytoskeleton associated proteins is critical in the maintenance of endothelial barrier integrity. In this process the role of PP1 has been well established, while the involvement of PP2A activity is less studied.

These cytoskeletal structures are controlled by various actin binding proteins, for instance plectin, spectrin, gelsolin, caldesmon, cofilin, HSP27 which are important in actin polymerization/depolymerization. Some of these proteins, such as cofilin, HSP27, caldesmon, are known to be dephosphorylated, therefore regulated by PP2A.

It is known that PP2A catalyzes the dephosphorylation and inactivation of CPI-17 in smooth muscle cells, thus prevents cell contraction. There is a growing body of evidence, showing the importance of PP2A activity in MT stabilization. A $\beta$ C holoenzyme was reported to be responsible for the dephosphorylation of brain tau. Similarly, our group revealed a substantial amount of PP2A associating with HSP27 and tau in MT-enriched fraction of pulmonary artery ECs. Nocodazole, which is a MT disrupting agent, induces gap formation and EC permeability increase. Inhibition of PP2A further enhanced nocodazole induced EC barrier compromise, referring to the role of PP2A in MT-mediated EC barrier regulation. In addition, the co-expression of the catalytic (PP2A C) and scaffolding (PP2A A) subunits considerably attenuated the thrombin or nocodazole induced EC barrier dysfunction.

## 2. AIMS

The catalytic and the structural subunits of PP2A are significantly involved in EC barrier preservation. However, the role of the regulatory B subunits of PP2A is unexplored. It is known that Ser/Thr phosphorylation regulates the AJ assembly, although the phosphatases involved in the dephosphorylation of AJ proteins are yet to be found. Phosphorylated CPI-17 is a potent inhibitor of MP, binding to its catalytic subunit makes the phosphatase unable to dephosphorylate its target, MLC, leading to cell contraction, and endothelial barrier dysfunction. PP2A dephosphorylates CPI-17, thus indirectly, but is involved in MLC phosphatase (MP) activation.

For these reasons specific goals of the presented work were the following:

- To further examine the functional significance of PP2A in endothelial cell barrier regulation focusing on the role of PP2A B $\alpha$ .
- To find new possible substrates or targets for PP2A in endothelial cells.
- To study mechanistic linkage between CPI-17 and EC cytoskeleton.
- To identify CPI-17 interacting partners in human lung microvascular endothelium (LMVEC).

### **3. MATERIALS AND METHODS**

#### ***Cell cultures and treatments***

Bovine pulmonary artery endothelial cells (BPAEC) were maintained in MEM supplemented with 10 % fetal bovine serum (FBS). Human pulmonary endothelial cells (HPAEC) and human lung microvascular endothelial cells (HLMVEC) were cultured in EBM-2 supplemented with 5% FBS and human embryonic kidney 293T (HEK 293T) cells were cultured in DMEM supplemented with 10 % FBS. Before any stimulation, cells were grown to 80-90% confluence. Cells were treated with 5 nM okadaic acid (90 min), 100 nM fostriecin (90 min), 50 nM thrombin (30 min) or with PMA (0,1  $\mu$ M for 30 min).

#### ***Immunofluorescence***

Cultured microvascular or macrovascular endothelial cells grown on 0.2% gelatin coated glass coverslips were washed with PBS and then fixed with 3.7% formaldehyde solution in TBS for 10 minutes and washed with TBS. The cells were permeabilized with 0.2% Triton X-100 in TBST for 5 min, washed with TBS, and blocked with 2% BSA in TBST for 1 hour. Cells were incubated with the primary, then the secondary antibodies diluted in blocking solution for one hour at room temperature. Actin microfilaments were stained with Texas red-labeled phalloidin for 1 hour at room temperature. After washing with TBS, coverslips were mounted with ProLong Gold Antifade medium.

#### ***Immunofluorescent microscopy***

After immunostaining, the glass slides were observed using a Nikon video-imaging system (Nikon Instech) consisting of a phase-contrast microscope Nikon Eclipse TE2000 connected to digital camera or with Zeiss Axiolab microscope using 63 x oil immersion objective. Confocal images were taken with an Olympus Fluoview FV1000 confocal microscope using UPLSAPO 60 x 1.35 NA oil immersion objective with images processed with FV10-ASW v1.5 software. Images were processed using PhotoShopCS5 Imaging software.

#### ***Image analysis of stress fiber formation***

Texas Red-stained EC monolayers treated with either thrombin or PP2A Ba siRNA were observed under Zeiss Axiolab microscope using 63 x oil immersion objective. 8 bit images were analyzed using Image J 1.46R. For valuation of stress fiber formation, F-actin fibers were marked out and the ratio of the cell area covered by stress fibers to the whole cell area was determined. At least 30 microscopic fields for each experimental condition were analyzed. The values were statistically analyzed by using GraphPad Prism 5 software.

### ***Expression plasmids and transfection protocols***

Plasmids encoding HSP27 pcDNA 3.1 V5/His, inhibitor 2 pcDNA 3.1 V5/His, and CPI-17 pcDNA 3.1 myc/His and PP2A B $\alpha$  pcDNA 3.1 V5/His construct were used in our experiments. HLMV (CPI-17 expression) cells grown until reached 80-90% confluence. Then cells were incubated with OPTI-MEM containing the appropriate amount of DNA and the X-tremeGENE HP transfection reagent for 6 hours in CO<sub>2</sub> incubator at 37°C. Then cells were incubated an additional 24-48 h and used for immunoprecipitation experiments. HEK (PP2A B $\alpha$ , inhibitor-2, HSP27 expression) cells were transfected at 80-90% confluence using 10  $\mu$ g DNA: 20  $\mu$ l PEI ratio. After 6 hours of transfection cells were incubated an additional 24-48 hours and used for pull down experiments.

### ***In vitro pull down assay***

Transfected HEK cells were rinsed with PBS, scraped and lysed in lysis buffer, and centrifuged (8,200 g) at 4°C. The supernatant was applied onto anti-V5-affinity gel and incubated for 4 hours at 4°C to bind recombinant B $\alpha$ , HSP27 or inhibitor 2. After centrifugation cleared BPAEC lysate was added onto the resin to which the recombinant B $\alpha$ , HSP27 or inhibitor 2 was bonded in advance, and incubated for 4 hours at 4°C. After centrifugation for 30 min at 8200 g, the resin was washed with PBS to remove unbound proteins, and then boiled with 2x SDS-sample buffer. These samples were further analyzed by Western blot.

### ***Immunoprecipitation***

HLMV cells 48 hours after transfection with CPI-17 construct, were washed with PBS and lysed with IP buffer. After centrifugation the supernatant was pre-cleared with protein G Sepharose at 4°C for 3 hours. The pre-cleared supernatant was incubated with EZ-view Red Anti c-myc Affinity gel. After overnight incubation at 4°C the supernatant was removed, and the remained beads were washed and resuspended in SDS sample buffer, then boiled for 5 min. The eluted samples were further analyzed by Western blot.

### ***Depletion of endogenous PP2A B $\alpha$ in endothelial cells***

To reduce the content of endogenous PP2A B $\alpha$  protein ECs (HLMVEC, HPAEC and BPAEC) were treated with SMART selection-designed PP2A B $\alpha$ -specific small interfering RNA (siRNA) duplex oligonucleotides, which guide sequence-specific degradation of the homologous mRNA. Non-targeting siRNA duplexes were used as controls (non-siRNA). Cells were transfected at 70-80 % confluence using DharmaFECT1 transfection reagent and used for further experiments after 48 hours post transfection.

### ***RNA isolation and RT-PCR***

Total RNA was isolated from endothelial cells and after reverse transcription the cDNA was used as a template in PCR reaction. Full-length PP2A B $\alpha$  was first cloned into pCR2.1-TOPO, then subcloned into pcDNA3.1/V5-His using *EcoRI* restriction site.

### ***Measurement of transendothelial electrical resistance (TER)***

The endothelial barrier properties were monitored using the highly sensitive biophysical assay with an electrical cell-substrate impedance sensing system, ECIS. HPAEC and HLMVEC were plated on small gold microelectrodes and culture media was used as electrolyte. Cells were transfected with small interfering RNA (siRNA) specific to the regulatory subunit of PP2A. After 72 hours incubation TER was measured in response to EC barrier disruptive agent (thrombin). Resistance data were normalized to the initial (steady state) voltage and plotted as a normalized resistance.

### ***SDS-PAGE, Western blot***

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After the separation by electrophoresis, proteins were transferred to nitrocellulose or to PVDF membranes using an electric current. The nonspecific binding sites of the membranes were blocked with incubation in 5% non-fat dry milk in TBST for 1 h and probed with the primary antibody of interest and then with HRP-conjugated secondary antibody. Immunoreactive proteins were detected on X-ray film with enhanced chemiluminescent detection system (ECL) reagents.

### ***Statistical analysis***

AxioVision LE64, Image J 1.46R and Photoshop CS6 were applied to edit immunofluorescent pictures. The analysis of ECIS results were done with Excel (Microsoft Corporation), and GraphPad Prism5 was used for graphical data presentation. To analyze Western blot results, autoradiograms were digitalized and the density of protein bands was analyzed by Image J 1.46R. All measurements are presented as the mean  $\pm$ SEM of at least 3 independent experiments. To compare results between each group, a 2-sample Student t-test was used. For comparison among different groups during ECIS data analysis I-way ANOVA tests were performed. Numbers of replicates and significant P values are indicated in the text or figures. Differences were considered statistically significant at P values below 0.05.

## 4. RESULTS

### 4.1. Role of PP2A in endothelial cell cytoskeleton organization

Human lung microvascular and bovine pulmonary artery endothelial cells were treated either with vehicle or with specific protein phosphatase 2A (PP2A) inhibitors: okadaic acid or fostriecin. The lack of PP2A activity was accompanied by the formation of F-actin stress fibers and the partial dissolution of the peripheral microtubules. Texas Red-phalloidin staining indicated the alteration of actin cytoskeleton, we found cortical F-actin dissolution and stress fiber formation reflecting a more contractile phenotype of cells. Besides the effect of PP2A inhibitors on microfilaments, tubulin staining revealed the partial depolymerization of the microtubules due to the treatments.

The inhibition of PP2A activity caused significant changes in the endothelial cytoskeleton. For this reason we intended to further clarify the role of the most abundant holoenzyme form of PP2A, the AB $\alpha$ C in the cytoskeleton arrangement. To reduce the level of the endogenous B $\alpha$  subunit in EC small interfering RNA technique was used. We performed reverse transcription (RT) PCR to verify the efficiency of PP2A B $\alpha$  silencing in mRNA level. To confirm our results on protein levels as well, we analyzed the transfected cells using Western blot. After the transfer, membranes were probed with a specific antibody against PP2A B and actin was also stained. Statistical analysis of our results confirmed 70-80% decrease in the endogenous level of PP2A B $\alpha$  compared to the non-silencing siRNA transfected cells with both methods. We further proved the silencing of the B subunit by immunofluorescent staining of PP2A B in EC monolayers transfected with PP2A B $\alpha$  specific siRNA or with non-siRNA.

We were also intended to investigate the effect of the depletion of B $\alpha$  on the actin cytoskeleton organization. In our previously published paper we showed that the overexpression of A and C subunits of PP2A significantly attenuated the thrombin or nocodazole induced EC barrier dysfunction. B $\alpha$  is the most widespread regulatory subunit of PP2A, however, its exact regulatory role in the endothelial cytoskeleton remains largely unexplored. To examine the direct involvement of the B $\alpha$  regulatory subunit in agonist induced EC barrier regulation, we challenged the PP2A B $\alpha$  depleted cells and non-siRNA transfected cells with thrombin. Thrombin treatment further enhanced the effect of PP2A B $\alpha$  depletion compared to non-siRNA transfected ECs. To further refine our results we conducted quantitative analysis of our pictures acquired by staining of actin microfilaments. The images were analyzed using Image J software. This analysis confirmed the increased stress fiber formation both in thrombin treated or in PP2A B $\alpha$  depleted cells. Overall our results further support previous findings from our group that PP2A plays a crucial role in the regulation of

endothelial cytoskeleton structure organization and B $\alpha$  is one of the PP2A regulatory subunits implicated in this function.

#### **4.2. PP2A B $\alpha$ depletion significantly enhances thrombin induced EC barrier loss**

Measurements of TER across human pulmonary EC monolayer after thrombin stimulation demonstrated a significant decrease in transendothelial resistance reflecting a dramatic EC barrier compromise. Previous data from our group confirmed the involvement of PP2A in EC barrier protection against edemagenic agonists, thrombin and nocodazole. In order to directly examine the role of PP2A B $\alpha$  subunit in EC barrier function, ECIS experiments were performed in PP2A B $\alpha$  depleted and non-siRNA transfected HPAE and in HLMVE cells. We examined the effect of PP2A B $\alpha$  on thrombin induced changes of EC permeability. Cells were treated with either vehicle, or thrombin followed by monitoring TER. Our results show that PP2A B $\alpha$  depletion decreases TER alone in HPAEC, but not in HLMVEC. Thrombin increases permeability of both cell types; furthermore the lack of PP2A B $\alpha$  significantly exacerbated the thrombin-induced decrease in TER and delayed or abolished TER recovery after thrombin. These findings indicate the direct involvement of PP2A B $\alpha$  in barrier protection against thrombin-induced EC barrier compromise.

#### **4.3. PP2A B $\alpha$ and adherent junctions**

To further investigate the role of the regulatory B $\alpha$  subunit of PP2A, we performed immunofluorescent staining to reveal its endogenous localization. Confocal images verified that the majority of PP2A B was localized in the cytoplasm, but it was also evident that a portion of B subunit co-localized with the cortical actin at the cell periphery. Cortical actin-like endogenous localization of PP2A B further reinforces our previous findings, that this PP2A targeting subunit is important in the cytoskeleton regulation suggesting the involvement of PP2A B in the cortical actin – cell junction assembly.

The partial membrane-like localization of the regulatory B subunit of PP2A led us to further examine the role of PP2A in the regulation of adherens junctions. Endothelial cell junctions and the regulation of their assembly by phosphorylation and dephosphorylation events are closely connected with EC permeability. The performing kinases are well studied, for instance it is well established that Ser/Thr phosphorylation of adherent junction protein,  $\beta$ -catenin, strengthens the VE-cadherin- $\beta$ -catenin interaction. However the role of Ser/Thr phosphatases remained unclear. To explore whether PP2A is directly involved in AJs regulation we performed pull down experiment to search for possible binding partners for PP2A among AJ proteins. We were able to identify adherent junction proteins along with the eluted B $\alpha$  using

specific antibodies against  $\beta$ -catenin, VE-cadherin, and phosphorylated  $\beta$ -catenin Ser552 providing evidence for the association of B $\alpha$  and EC adherent junction proteins.

#### **4.4. PP2A B $\alpha$ regulates the dephosphorylation of $\beta$ -catenin and affects AJ assembly**

Our previous experiment showed specific binding between PP2A and AJs proteins: VE-cadherin, total and phosphorylated  $\beta$ -catenin. To learn more about the role of PP2A in the regulation of cell junction assembly/disassembly, we employed okadaic acid or fostriecin, on micro and macrovascular endothelial cells. Fostriecin and okadaic acid treated cells showed an increase in the phosphorylation level of Ser552 side chain in  $\beta$ -catenin, which suggested that PP2A may have a role in the regulation of  $\beta$ -catenin dephosphorylation. In addition OA and fostriecin treated EC monolayers were immunostained for adherent junction proteins VE-cadherin,  $\beta$ -catenin. Both PP2A inhibitors induced interruption of continuous VE-cadherin staining at the cell periphery suggesting disruption of AJs. At the same time  $\beta$ -catenin staining at the edge of the cells was less pronounced after OA treatment without evident decrease of total protein amount suggesting some cellular redistribution of  $\beta$ -catenin. The effect of specific PP2A inhibition on the subcellular distribution of phospho- $\beta$ -catenin was also examined. Immunofluorescent staining of EC using specific antibody revealed that  $\beta$ -catenin phosphorylated on Ser552 translocated from the cell membrane to the cytoplasm.

Our results indicated that PP2A inhibition negatively affected the adherens junction assembly and the lack of PP2A activity enhanced the phosphorylation of  $\beta$ -catenin. Furthermore we showed direct interaction between PP2A B $\alpha$  and AJs proteins. Next we wanted to examine the effect of specific depletion of PP2A B $\alpha$  on AJs. We hypothesized that the regulatory subunit targets PP2A to the cell periphery where it is able to interact with  $\beta$ -catenin. We performed Western blot experiments using PP2A B $\alpha$  depleted cells and non-siRNA transfected cells. Similarly to the effects of okadaic acid and fostriecin, the lack of B $\alpha$  regulatory subunit significantly elevated the level of phospho- $\beta$ -catenin Ser552. In addition we observed an inverse correlation between the protein levels of B $\alpha$  and the phosphorylation level of phospho-S552- $\beta$ -catenin. Membranes were probed for PP2A B to confirm the efficiency of the silencing. According to the statistical analysis of the Western blot, the depletion was about 70 %. In the next set of experiments, immunofluorescent staining was performed on PP2A B $\alpha$  depleted and non-siRNA transfected BPAEC. The monolayers were double stained for  $\beta$ -catenin and for phospho- $\beta$ -catenin Ser552. Depletion of PP2A B $\alpha$  led to the translocation of  $\beta$ -catenin from the cell membranes to the cytoplasm and caused the disruption of AJs. Interestingly, almost the entire amount of phospho Ser552- $\beta$ -catenin in PP2A B $\alpha$ -depleted cells



was located in the cytoplasm, but not at the cell edges. These data suggested that PP2A B $\alpha$  may be important for integrity of AJs, which is consistent with our data indicating that depletion of this subunit leads to EC barrier compromise. Overall, these data indicate the involvement of PP2A in the regulation of  $\beta$ -catenin phosphorylation level and cellular distribution, which affect AJs assembly and EC permeability.

#### **4.5. The role of a PP2A target, CPI-17, in endothelial cell barrier regulation**

##### ***Investigation of putative binding partners for CPI-17***

CPI-17 is known as a specific inhibitor of MP which is involved in the endothelial cytoskeleton and barrier regulation. Phosphorylation of CPI-17 at Thr38 by PKC enhances its inhibitory potency ~1000-fold, thus converting the protein into a potent inhibitor of MP. Data of literature suggested that PP2A can be potentially involved in MP activation via dephosphorylation/deactivation of CPI-17, in consequence opposing EC contraction and permeability increase. The expression of endogenous CPI-17 in human lung microvascular endothelial cells was shown and its involvement in the regulation of EC cytoskeleton and permeability. Besides the importance of CPI-17 in EC barrier regulation little is known about its interacting partners in the EC cytoskeleton. Putative binding partners for CPI-17 in lung endothelium using bacterial two-hybrid library screening system were identified. Human cDNA library was screened to find candidates as CPI-17 interacting partners and fourteen proteins were identified as potential binding partners for CPI-17. Five of them plectin 1 isoform 1, alpha II spectrin, OK/SW-CL.16, gelsolin isoform a and junction plakoglobin are known to have function in actin cytoskeleton organization and cell adhesion. To confirm the newly found interacting partners of CPI-17, immunoprecipitation (IP) was performed in microvascular endothelial cells. HLMVEC were transfected with CPI-17 pcDNA myc/His construct. In the following Western blot experiment anti-myc antibody was used to confirm the success of the IP, and antibodies against plectin, spectrin, gelsolin, and plakoglobin were used to detect each protein which was assumed as a putative binding partner from the previous bacterial two-hybrid screening. The nine remaining candidates were excluded from this study due to their lesser relevance in the cytoskeletal regulation. We could confirm the interaction between overexpressed CPI-17 and plakoglobin by IP. Plakoglobin is an adherens junction protein, highly homologous to  $\beta$ -catenin playing an important role in cadherin/catenin complex assembly. PKC activator PMA evokes the phosphorylation of CPI-17 on Thr38. We found that the binding affinity between CPI-17 and plakoglobin was decreased in the cells that were pretreated with PMA. Next, to further confirm the specificity of the plakoglobin CPI-17 interaction, another set of IP experiments were conducted. IP samples were probed not only for

plakoglobin, but also for  $\beta$ -catenin, VE-cadherin and p120, however, only binding of plakoglobin was detected, further demonstrating the specificity of CPI-17-plakoglobin interaction. Interestingly, MYPT1, targeting subunit of MP was not found as a binding partner with the bacterial two-hybrid screening, however, it was co-immunoprecipitated with the overexpressed CPI-17 as it was expected.

#### ***Co-localization of plakoglobin and CPI-17 in HLMVEC***

To further investigate the interaction between CPI-17 and plakoglobin, immunofluorescent staining was employed. CPI-17 pcDNA myc/His or empty vector transfected HLMVEC monolayers were both treated with vehicle or with PMA and stained for plakoglobin or c-myc. We could detect co-localization of recombinant CPI-17 and endogenous plakoglobin at the attachment sites of neighboring cells. Although, increased plakoglobin staining was observed in PMA treated cells, the CPI-17 staining is rather become scarce in the cell membrane.

These stainings provided further evidences of CPI-17, plakoglobin interaction in untreated conditions where inactive CPI-17 does not affect MP and AJs are stable. In turn, we could not detect co-localization between these two proteins in PMA treated cells suggesting that activated CPI-17 is involved in different signaling pathways.

Taken together using immunofluorescence staining, Western blot, specific siRNA depletion, pull-down assay and immunoprecipitation and transendothelial resistance measurement we could further strengthen our previous findings that PP2A is intimately involved in the regulation of endothelial cytoskeleton structure and in the regulation of adherens junction assembly. Moreover we could identify plakoglobin, a new interacting partner for a PP2A substrate, CPI-17 and examined its role in the endothelial barrier regulation.

## 5. SUMMARY

Endothelial barrier integrity is dependent on Ser/Thr protein phosphorylation of cytoskeletal and adherens junction proteins. Protein phosphatase 2A (PP2A) is involved in the maintenance of barrier integrity and myosin phosphatase (MP) is important in the regulation of actomyosin contraction. Our aim was to further explore the role of PP2A in the EC barrier regulation, and to find binding partners of the PP2A substrate, CPI-17, an endogenous inhibitor of MP.

We examined macro- and microvascular EC and provided new evidence on the significance of PP2A in the regulation EC cytoskeleton and adherens junction (AJ) assembly with special focus on the B $\alpha$  regulatory subunit. Inhibition of PP2A with OA and fostriecin affected actin arrangement and caused microtubule dissolution. Depletion of the B $\alpha$  subunit had similar effect, as it led to actin reorganization. Moreover the lack of PP2A B $\alpha$  significantly exacerbated thrombin-induced increase in EC barrier permeability. AJ proteins VE cadherin and  $\beta$ -catenin were detected as binding partners of B $\alpha$  with pull down assay. Inhibition of PP2A caused the redistribution of  $\beta$ -catenin and VE-cadherin, from the cell junctions to the cytosol and nucleus and resulted in phosphorylation of  $\beta$ -catenin. Similarly, the depletion of B $\alpha$  affected the F-actin organization in EC, and lead to the disruption of AJs, parallel with  $\beta$ -catenin phosphorylation on Ser552. The effect of B $\alpha$  depletion was further enhanced by thrombin treatment.

It is established that PP2A dephosphorylates, thus inactivates CPI-17, however little is known about the linkage of CPI-17 phosphorylation and endothelial cytoskeleton. Phosphorylation of CPI-17 at Thr38 by PKC enhances its inhibitory potency and converts the protein into a potent inhibitor of MP. CPI-17 is expressed in HLMVEC and it was found to be involved in the regulation of EC barrier. Plakoglobin, an AJ protein stabilizing cell junctions, was revealed as putative binding protein of CPI-17 by two-hybrid screening. We confirmed the plakoglobin-CPI-17 interaction in HLMVEC, furthermore we detected decreased association between these two proteins by PMA-induced CPI-17 phosphorylation.

Our results suggests that PP2A B $\alpha$  has a functional role in the barrier protective function of PP2A and in the regulation of AJ proteins via the dephosphorylation of  $\beta$ -catenin. Moreover, our data suggest that CPI-17 can interact with major elements of cytoskeleton through its binding partner plakoglobin and have a possible regulatory role in EC barrier function.

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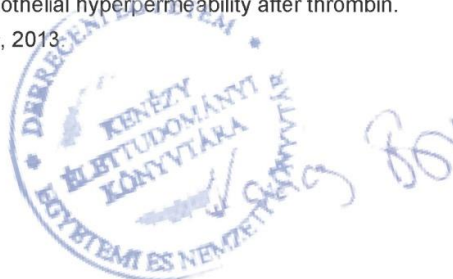
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### List of publications related to the dissertation

1. **Kása, A.**, Czikora, I., Verin, A.D., Gergely, P., Csontos, C.: Protein phosphatase 2A activity is required for functional adherent junctions in endothelial cells.  
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DOI: <http://dx.doi.org/10.1016/j.mvr.2013.05.003>  
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2. Kim, K., Adyshev, D.M., **Kása, A.**, Zemskov, E.A., Kolosova, I.A., Csontos, C., Verin, A.D.: Putative protein partners for the human CPI-17 protein revealed by bacterial two-hybrid screening.  
*Microvasc. Res. 88, -24, 2013.*  
DOI: <http://dx.doi.org/10.1016/j.mvr.2013.04.002>  
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3. Adyshev, D.M., Dudek, S.M., Moldobaeva, N., Kim, K., Ma, S., **Kása, A.**, Garcia, J.G.N., Verin, A.D.: Ezrin/radixin/moesin proteins differentially regulate endothelial hyperpermeability after thrombin.  
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## **Presentation related to the dissertation**

1. PhD Symposium of the Doctoral School of Molecular Medicine 2009  
*Investigation of Protein phosphatase 2A regulatory subunits in vascular endothelial cells*
2. PhD Symposium of the Doctoral School of Molecular Medicine 2010  
*The role of protein phosphatase 2A (PP2A) regulatory subunits in the regulation of pulmonary endothelial cell (EC) cytoskeleton structure*
3. Vascular Biology Center Seminar 2013, Georgia Regents University, Augusta, GA, USA  
*Ser/Thr protein phosphatases in lung endothelial barrier regulation*

## **Poster presentations related to the dissertation**

1. Anita Kása, István Czikora, Pál Gergely, Csilla Csontos: Investigation of PP2A regulatory subunits in endothelial cells  
38th. Membrane-Transport Conference 2008, Sümeg
2. **Anita Kása**, Anita Boratkó, István Czikora, Pál Gergely, Csilla Csontos: Characterization of the regulatory subunits of protein phosphatase 2A in pulmonary endothelial cells.  
Conference of the Hungarian Biochemical Society 2008, Szeged, August 31-September 03, 2008
3. **Anita Kása**, István Czikora, Pál Gergely, Csilla Csontos: The role of Protein phosphatase 2A regulatory subunits in the control of endothelial cell cytoskeleton structure  
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4. **Anita Kása**, István Czikora, Pál Gergely, Csilla Csontos: The role of the B subunits of PP2A in the regulation of cytoskeletal structure of pulmonary endothelial cells  
Conference of the Hungarian Biochemical Society 2009, Budapest
5. **Anita Kása**, Pál Gergely, Csilla Csontos: Investigation the role of Protein phosphatase 2A (PP2A) regulatory subunits in the control of cell junctions in endothelium  
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6. István Czikora, Kyung-mi Kim, **Anita Kása**, Bálint Bécsi, Alexander D. Verin, Pál Gergely, Ferenc Erdódi, Csilla Csontos: Characterization of the effect of TIMAP phosphorylation on its interaction with protein phosphatase 1  
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7. **Anita Kása**, Pál Gergely, Csilla Csontos: The role of Protein phosphatase 2A in the regulation of  $\beta$ -catenin dephosphorylation in ECs

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8. **Anita Kása**, Csilla Csontos, Pál Gergely, and Alexander D. Verin:  $\beta$  regulatory subunit targets PP2A to Adherens Junctions and regulates the dephosphorylation of  $\beta$ -catenin in endothelial cells

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9. **Anita Kása**, Pál Gergely, Alexander D. Verin, Csilla Csontos.: Phosphatase 2A is involved in Adherens Junction Regulation in Endothelial Cells

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10. **Anita Kása**, Kyung-mi Kim, Djanybek Adyshev, Evgeny A. Zemskov, Csilla Csontos, Alexander D. Verin: CPI-17 Associates With Adherens Junction Protein Plakoglobin And Regulates EC Permeability

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