

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

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

ANITA KÁSA

Supervisor: Csilla Csontos, Ph.D.



UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2013

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2. ABBREVIATIONS

AJ – Adherens Junction

ALI – Acute Lung Injury

ARDS – Acute Respiratory Distress Syndrome

ATCC - American Type Culture Collection

BPAEC - Bovine Pulmonary Artery Endothelial Cells

BSA - bovine serum albumin

cDNA - complementary DNA

CPI-17 - PKC potenciated inhibitory protein of 17 kDa

DMEM - Dulbecco's Modified Eagle Medium

dNTP - Deoxyribonucleotide triphosphate

DMSO - dimethyl sulfoxide

ECs - endothelial cells

ECIS - Electric Cell Impedance Sensing System

ECL - Enhanced Chemiluminescence

EDTA - Ethylene diamine tetraacetic acid

EGTA - ethylene glycol tetraacetic acid

EGM-2-MV - Endothelial Growth Medium for microvascular cells

FCP/SCP - TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase

FBS - fetal bovine serum

GA - Gentamicin Sulfate and Amphotericin-B

HEK - Human Embryonic Kidney

HLMVEC - Human Lung Microvascular Endothelial Cells

HPAEC - Human Pulmonary Artery Endothelial Cells

IL - interleukin

mAb- monoclonal antibody

MEM - Minimum Essential Medium

MLC – myosin light chain

MP – Myosin Phosphatase

MYPT1- Myosin targeting subunit 1

OA - octadecanoic acid

pAb- polyclonal antibody
PBS - phosphate buffered saline
PEI - polyethilenimine
PKA - Protein Kinase A
PKC - Protein Kinase C
PMA - phorbol 12-myristate 13-acetate
PMSF - phenyl-methyl-sulphonyl-fluoride
PP1 - Protein phosphatase 1
PP2A - Protein phosphatase 2
PP2B - Protein phosphatase 2B
PVDF - Polyvinylidene difluoride
SDS - Sodium Dodecil Sulfate
TER – transendothelial electrical resistance
TNF α – tumor necrosis factor α
TJ – tight junction
TBS - Tris buffered saline
VEGF - Vascular Endothelial Growth Factor

3. INTRODUCTION

Lung endothelium regulates movement of fluid and macromolecules between the blood and the interstitium. Disruption of endothelial barrier results in the movement of fluid and macromolecules into the interstitium and pulmonary air spaces causing pulmonary edema (Pugin, 1999) which is a common feature of Acute Lung Injury (ALI) and its more severe form Acute Respiratory Distress Syndrome (ARDS). Despite the importance of the endothelial cell (EC) barrier little information exists concerning the mechanisms involved in preservation of barrier integrity. However, it is clear that the regulation of the pulmonary EC barrier function is largely dependent upon the reversible phosphorylation of Ser/Thr residues of EC cytoskeleton proteins as well as proteins of cell junctions (Bazzoni and Dejana, 2004; Dejana, 1999; Dudek and Garcia, 2001).

PP2A is a major Ser/Thr protein phosphatase responsible for dephosphorylation and regulation of many molecular targets. The core dimer consists of a catalytic (PP2A C) and a structural subunit (PP2A A). This dimer binds the third variable regulatory subunit (generally called PP2A B subunit), which is responsible for the subcellular localization and the substrate specificity of the enzyme. B α and the AB α C trimer form of PP2A was demonstrated to play several specific functional roles in the regulation of the cytoskeleton (Janssens et al., 2008; Sontag et al., 1999; Turowski et al., 1999).

Adherens junctions (AJs) present the majority of cell junctions maintaining the EC barrier. AJs are composed of VE-cadherin and its cytoplasmic binding partners: p120, α -, β -catenin, which link AJs to the actin cytoskeleton. The assembly of VE- cadherin-catenin complex is regulated by Ser/Thr phosphorylation, and their dissociation leads to EC barrier dysfunction (Huber and Weis, 2001). Up to now, very limited information is available regarding the functional link between PP2A and AJs, and the mechanisms of PP2A-mediated maintenance of AJs and EC barrier are still unknown.

Myosin phosphatase, MP, is a type-1 protein phosphatase. It is directly involved in EC barrier preservation via dephosphorylation of MLC. It was shown that MP activity is inhibited by Rho-dependent phosphorylation of its regulatory subunit, MYPT1 (Somlyo and Somlyo, 2000). CPI-17, a soluble globular protein is an inhibitor of MP activity (Eto et al., 1995). Phosphorylation of CPI-17 at Thr38 by PKC enhances its inhibitory potency. Therefore CPI-17 may represent an alternative mechanism for inhibition of MP different from the one via phosphorylation of MYPT1. Importantly, CPI-17 is a cytoskeletal target of PP2A, as PP2A can

dephosphorylate and inhibit CPI-17 activity towards MP (Ito et al., 2004). It is already known that CPI-17 is involved in the regulation of EC permeability (Kolosova et al., 2004), however, the direct link between PP2A-mediated CPI-17 dephosphorylation and EC barrier regulation is unknown.

In our studies we intended to identify further linkage between the changes in EC cytoskeleton structure and PP2A activity, as well as to examine the molecular mechanisms of PP2A-induced barrier preservation, focusing on PP2A-induced dephosphorylation of AJ proteins, mainly β -catenin, and the regulation of MP activity and AJ structure by the MP inhibitor CPI-17, which is dephosphorylated/regulated by PP2A.

4. REVIEW OF THE LITERATURE

4.1. The lung vascular barrier

4.1.1. 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 (Mehta and Malik, 2006). Mechanisms which govern the highly clinically relevant process of increased endothelial permeability are under intense investigation. The vasculature is lined by heterogeneous population of endothelial cells. This heterogeneity is derived from the origin of endothelial cells in the vascular tree. The barrier function, surface biochemistry, and morphology of confluent monolayers of microvascular and macrovascular endothelial cells were compared in Dr. Schnitzer's work. They found that the permeability characteristics were different for these two cell types. Permeability was ~16-fold less for sucrose and to 2-fold less for albumin in microvascular EC (MVEC) compared to macrovascular EC monolayers. Furthermore, electron microscopy revealed that MVEC have more developed intercellular junctions with more focal membrane adhesion sites per junction than the macrovascular cells (Schnitzer et al., 1994). Pulmonary artery endothelial cells (macrovascular ECs) play important physiological and pathophysiological roles in the human body. For example, they participate in blood homeostasis, blood-tissue exchange regulation under various conditions (Terramani et al., 2000). 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 (Kelly et al., 1998b).

4.1.2. Clinical and physiological importance of endothelial barrier in the lung

The alveolar-capillary barrier is formed by the microvascular endothelium, the alveolar epithelium and the basement membrane (Figure 4.1). 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). Despite recent therapeutic advances, these conditions still have high rates of patient mortality. 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 (Pugin et al., 1999). Neutrophils are adhering to the injured endothelium and migrating through the interstitium into the alveoli (Anderson and Thielen, 1992; Bachofen and Weibel, 1982), whereas the macrophages are secreting cytokines (IL-1, 6, 8 and 10) and $\text{TNF}\alpha$ (Matthay, 1999). ALI/ARDS may also lead to impaired gas exchange and may cause respiratory failure (Lewis and Jobe, 1993). 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. Apart from ventilation strategies there is no standard treatment for pulmonary edema, making the investigation of regulatory mechanisms of endothelial barrier dysfunction important (Ware and Matthay, 2000).

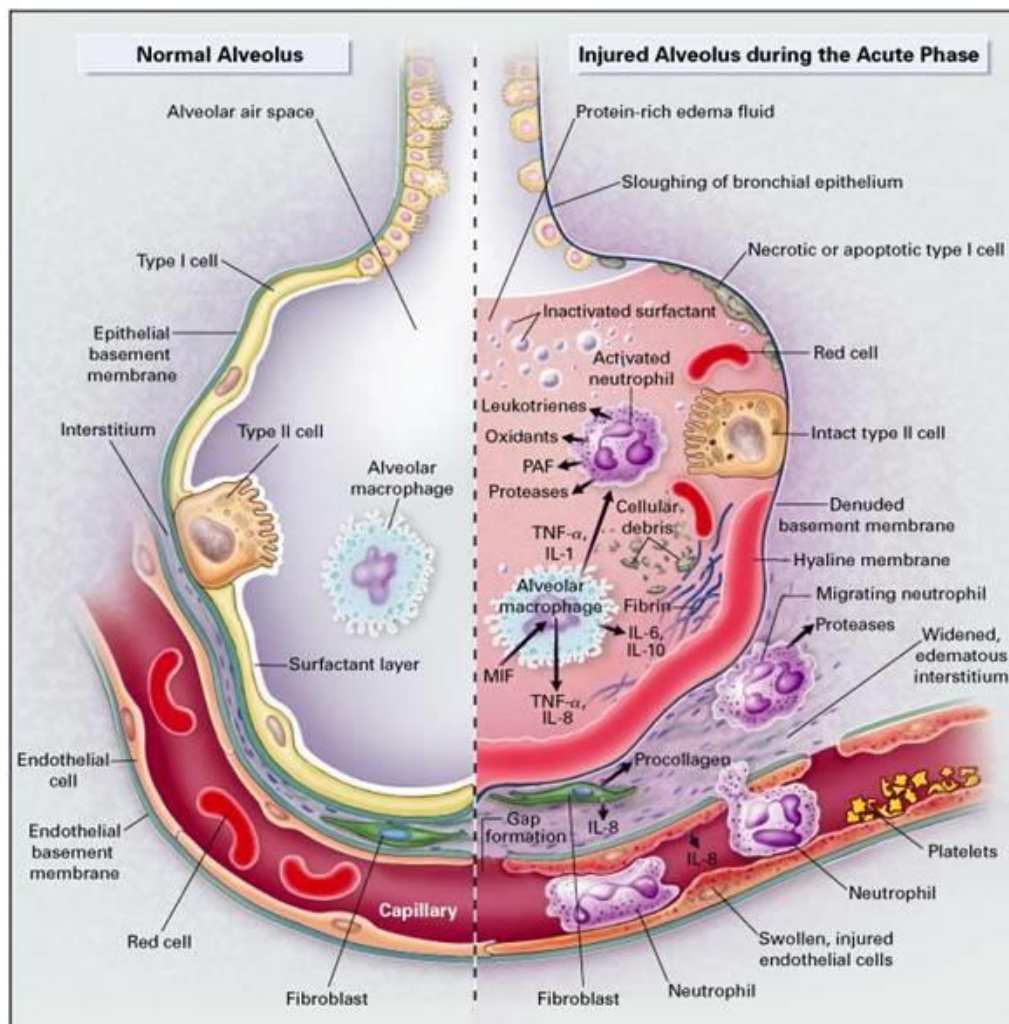


Figure 4.1. The normal alveolus and the injured alveolus in the acute phase of acute lung injury and the acute respiratory distress syndrome (Ware and Matthay, 2000).

4.2. Mechanisms regulating endothelial permeability

4.2.1. Endothelial barrier properties

The semi-permeable endothelium shows heterogeneity in the pulmonary vasculature. It enables to regulate the flux of fluid and solutes between the blood and the interstitium. 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 (Feletou, 2011). Under non-pathological conditions EC permeability is a well regulated physiological exchange of materials between the blood and the underlying interstitium. The majority of macromolecules are transported through the macrovasculature (Lamm et al., 1988). Blum and coworkers showed that primary cultures of microvascular endothelial monolayers produced 10 times higher transmonolayer electrical resistance (TER) than that of macrovascular EC (Blum et al., 1997). Although the precise mechanisms that regulate this variability are still under investigation, microarray analysis showed a significant variation in microvascular and macrovascular gene expression (Chi et al., 2003). Extracellular matrix proteins, collagen 4 α 1, collagen 4 α 2, and laminin were associated with microvessel endothelia, while fibronectin, collagen 5 α 1, and collagen 5 α 2 were seen with the large vessel endothelia (Chi et al., 2003).

4.2.2. Endothelial permeability pathways

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 and epithelial cell monolayers can involve transcellular, paracellular or the combination of both pathways (Figure 4.2). However, majority of trafficking occurs through the paracellular pathway. The transcellular transport involves membrane-attached and cytosolic caveolae that migrate through the endothelial cells and transfer macromolecules from the blood to the interstitium (Mehta and Malik, 2006). The main player in this process is the Src kinase, which can phosphorylate caveolin-1 on tyrosine residues inducing the migration of the vesicles across the endothelium (Rothberg et al., 1992).

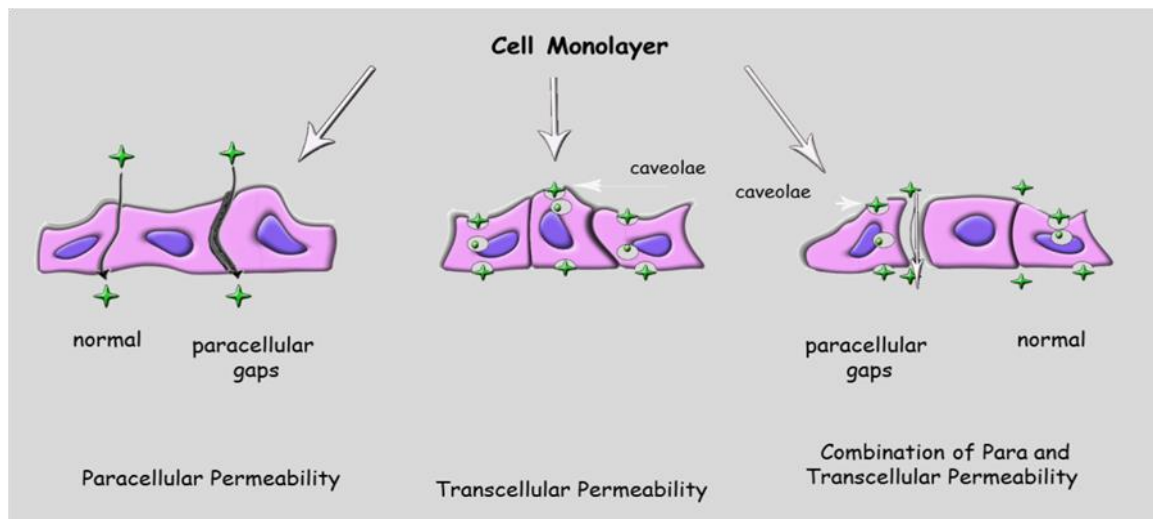


Figure 4.2. Major pathways of endothelial cell permeability

The capillary endothelium is impermeable to macromolecules under basal conditions. This is due to the network of cytoskeletal and cell-junction elements which will protect endothelial barrier integrity. In state of acute or chronic inflammation, sepsis, diabetes, angiogenesis or tumor metastasis, mediators such as histamine, serotonin, thrombin, bradykinin, substance P, platelet activating factor, cytokines, VEGF and reactive oxygen species induce the loss of endothelial barrier function, which will lead to permeability increase to solute and plasma proteins via the paracellular pathway (Feletou, 2011).

4.2.3. Permeability increasing agonists

During inflammation the endothelium is activated by mechanical alterations (excessive level of stretch or shear stress), by edemagenic agonists such as thrombin, histamine, tumor necrosis factor (TNF) or reactive oxygen species (Dudek and Garcia, 2001). Thrombin is a serine protease generated by injured endothelial cells by the cleavage of circulating prothrombin, participating in the prothrombinase complex, containing Factors X and V, Ca^{2+} and membrane phospholipids (Grand et al., 1996). Thrombin not only induces coagulation, but also affects endothelial barrier function by releasing of inflammatory mediators and growth factors as well as inducing leukocyte adhesion on EC surface (Bogatcheva et al., 2002). Thrombin binds to G-protein-coupled protease activated receptors (PAR-1, PAR-2, and PAR-3) and is implicated in several cellular effects (Kawkitinarong et al., 2004) and it is an important factor in the development of ALI and ARDS. The *in vitro* effects of thrombin has been well established, it can increase EC albumin permeability and decrease transendothelial electrical

resistance (TER), reflective of EC barrier dysfunction and cytoskeleton rearrangement (Garcia et al., 1995; Garcia et al., 1986).

4.2.4. Regulation of endothelial paracellular gap formation

Endothelial barrier integrity is maintained by the precisely regulated balance between the actomyosin contractile forces and adhesive cell-cell, cell-matrix tethering forces (Dudek and Garcia, 2001; Garcia and Schaphorst, 1995). Both competing forces are tightly linked to the cytoskeleton comprising actin microfilaments, microtubules and intermediate filaments. The complex network of cytoskeleton is critical in the EC barrier regulation. For example, Shasby and coworkers challenged ECs with cytochalasin D, a well-known actin filament disrupting agent, and observed endothelial permeability increase and paracellular gap formation (Shasby et al., 1982). Regulation of EC barrier function is critically dependent upon activities of protein kinases and protein phosphatases (Csontos et al., 2007). Specifically, cell junction proteins, cytoskeletal, or cytoskeleton-associated proteins are regulated by the precise mechanism of phosphorylation/dephosphorylation events. Under basal conditions, when the balance is tilted towards tethering forces, a thick cortical actin ring can be observed, where endothelial cells can maintain tight connections with each other and the underlying matrix (Dudek and Garcia, 2001). Due to the effect of barrier compromising agents, for example thrombin, contractile forces dominate. Thrombin cleaves its receptor (PAR-1), causing elevation of intracellular Ca^{2+} , which will activate the Ca^{2+} /calmodulin dependent myosin light chain kinase (MLCK), that will phosphorylate MLC and consequently actomyosin interaction and contraction will be evoked (Tinsley et al., 2000; Wysolmerski and Lagunoff, 1991) (Figure 4.3.)

Thrombin also activates the small GTPase Rho signaling pathway which results in barrier dysfunction (Wojciak-Stothard and Ridley, 2002). GTP bound Rho activates its downstream effector, Rho-kinase, which phosphorylates the myosin phosphatase targeting subunit (MYPT1) of myosin phosphatase (MP), at the inhibitory Thr696 and Thr853 sites which leads to the inhibition of MP, and accumulation of diphospho-MLC and cell contraction (Birukova et al., 2004b).

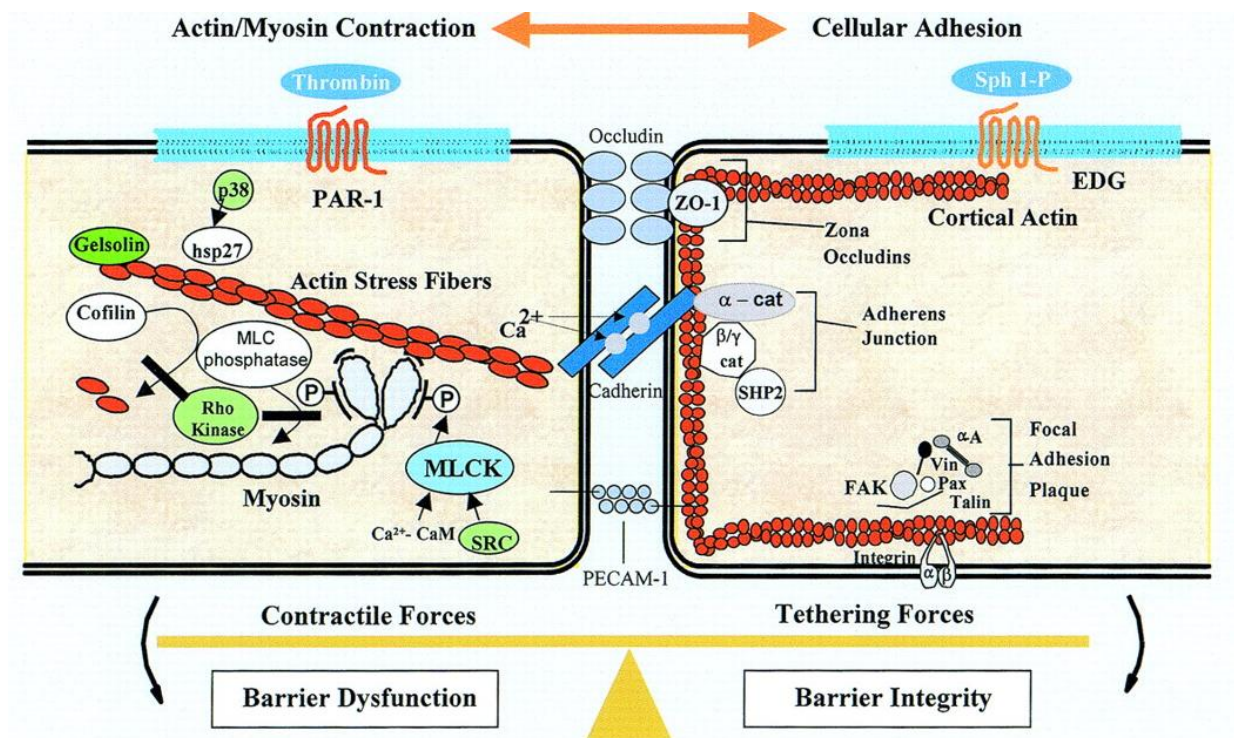


Figure 4.3. Actomyosin contractile elements and cellular adhesive forces regulate endothelial paracellular gap formation (Dudek and Garcia, 2001).

4.3. Cell junctions in the endothelium

The vascular endothelium is constantly exposed to hemodynamic stimuli, such as shear stress, contraction or dilation of the vessels. The continuous reorganization of cell junctions and the cytoskeleton have key importance in the maintenance of the endothelial barrier integrity. Reshaping of the cells allows the endothelial monolayer to adapt to the dynamic conditions to which it is exposed (Dejana et al., 2009). Communicating structures like adherens junctions (AJ) and tight junctions (TJ) link ECs together and regulate endothelial permeability, as part of the paracellular pathway. Gap junctions (GJ) are also present in ECs and play an important role in endothelial functions; however, there is no literature data about their involvement in EC permeability. AJs and TJs have different functions. AJs are responsible for maintaining cell-cell contacts while TJs regulate the transport of ions and solutes through the paracellular pathway. Importantly, both structures can transfer intracellular signals and control many endothelial cell functions (Bazzoni and Dejana, 2004) (Figure 4.4). Adherens junctions represent the majority of cell junctions comprising the endothelial barrier, in contrast with epithelial cells where tight junctions dominate (Mehta and Malik, 2006). Endothelial cells contain vascular endothelial (VE)-cadherin as a transmembrane protein that mediates hemophilic binding of adjacent cells.

The extracellular region contains five repeating domains which coordinate with calcium ions and form a rod-like structure. The intracellular tail of VE-cadherin has two domains, the juxtamembrane domain (JMD) and the C-terminal domain (CTD). JMD binds p120 catenin, while CTD binds β -catenin or plakoglobin (alternative name: γ -catenin) which attach α -catenin to link the cadherin–catenin complex to the actin cytoskeleton. α -catenin also interacts with other actin-binding proteins, specifically, α -actinin, vinculin, tight junction zonula occludin proteins: ZO-1, ZO-2 and possibly spectrin. VE-cadherin function is necessary for the proper assembly of AJs, and for normal endothelial barrier function (Stevens et al., 2000). VE-cadherin impairing results in interstitial edema and inflammation in lung and heart microvasculature (Corada et al., 1999).

Catenins also play an important role in the regulation of AJ assembly. β -catenin has a dual role in cells. First it was identified as a component of AJs in the late '80s. Kemler and colleagues could isolate β -catenin together with α -catenin and plakoglobin (Valenta et al., 2012). Later genetic and embryogenic studies revealed β -catenin as a part of the Wnt signaling pathway playing an important role in embryonic development and tumorigenesis (Kikuchi, 2003; Nusslein-Volhard and Wieschaus, 1980). Plakoglobin is closely related to β -catenin, sharing 80% sequence identity (Wallez and Huber, 2008) and can bind the cytoplasmic domains of the classical cadherins. Furthermore, it is important in plakoglobin/desmosomal cadherin interactions. Both β -catenin and plakoglobin were shown to stabilize the linkage between VE-cadherin and the actin cytoskeleton, thus regulating endothelial barrier function (Mehta and Malik, 2006).

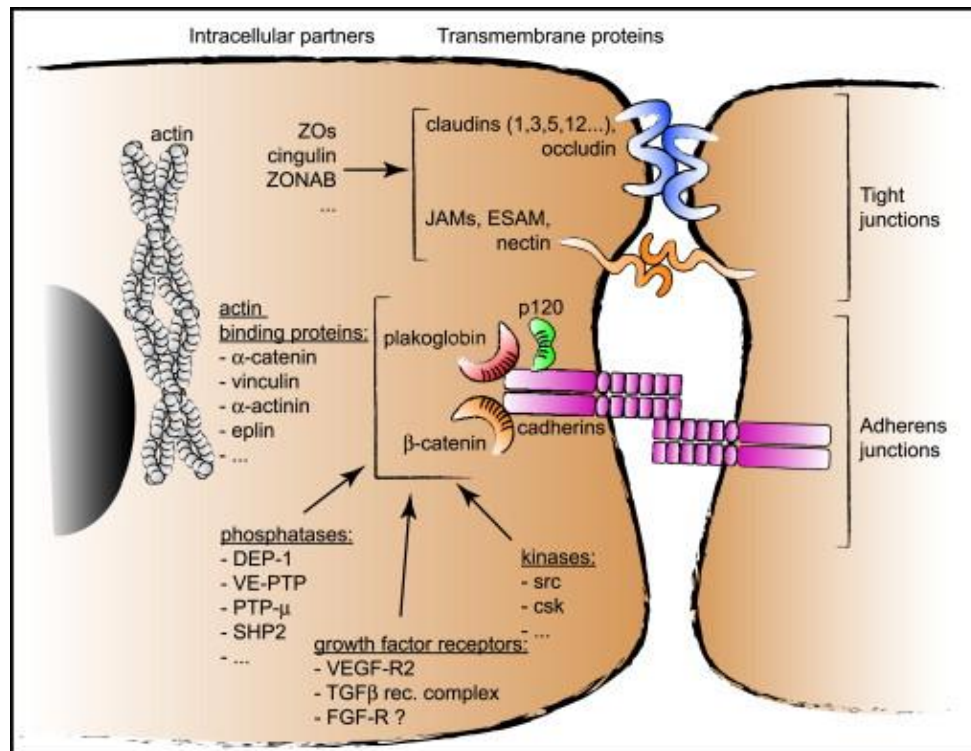


Figure 4.4. Schematic representation of tight junctions and adherens junctions in endothelial cells (Dejana et al., 2009)

4.3.1. 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. Histamine, tumor necrosis factor (TNF) and vascular endothelial growth factor induced tyrosine phosphorylation of VE-cadherin, β -catenin and p120 has long been studied. It was shown that these events increase endothelial barrier permeability (Dejana et al., 2008). Recent studies revealed the possibility of AJ regulation by Ser/Thr phosphorylation as well. For instance, alterations in EC permeability due to increased concentrations of thrombin, histamine or growth factors were mediated by the phosphorylation of MLC and by the activation of p21-activated kinase (PAK), a Ser/Thr kinase (Stockton et al., 2004). Huber and Weis identified cadherin residues (Ser684 and Ser699) phosphorylated by casein kinase II (CKII) - and GSK-3 β , later even more Ser residues were recognized. This phosphorylation of cadherin could stabilize and strengthen the catenin-cadherin complex several hundred fold (Choi et al., 2006; Huber and Weis, 2001; Sampietro et al., 2006). However, there are some reports indicating that cadherin phosphorylation can be a negative factor for binding to β -catenin (Dupre-Crochet et al., 2007). In addition, β -catenin, as a component of the Wnt-signaling pathway, directly associates with casein kinase I (CKI) and

GSK-3 β . These kinases induce the phosphorylation of β -catenin on Ser33/37 and Thr41, respectively, leading to its ubiquitination and proteosomal degradation (Aberle et al., 1997; Rubinfeld et al., 1996). Protein kinase A (PKA) was also shown to phosphorylate β -catenin at Ser552 and Ser675 sites (Fang et al., 2007; Taurin et al., 2006) which induce β -catenin transcriptional activity.

Despite the significance of these proteins' phosphorylation the phosphatases which make the process reversible are still unknown. Only a few data suggest that the Ser/Thr phosphatases may have a role in the regulation of AJ proteins. For example okadaic acid and calyculin A, protein phosphatase 1 and 2 inhibitors caused hyperphosphorylation of β -catenin leading to the disruption of cell junctions in epidermal cells (Serres et al., 1997). The above mentioned data underline the significant role of adherens junctions and their phosphorylation in EC permeability regulation. Also they highlight the importance to elucidate the role of protein phosphatases in endothelial cell junction assembly.

4.4. 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 (Cohen, 1997; Jackson and Denu, 2001; Kelly et al., 1998a; Shi, 2009). Reversible phosphorylation of proteins is driven by opposing activities of protein kinases and protein phosphatases (Figure 4.5). Protein kinases catalyze the transfer of terminal γ -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 (Shi, 2009).

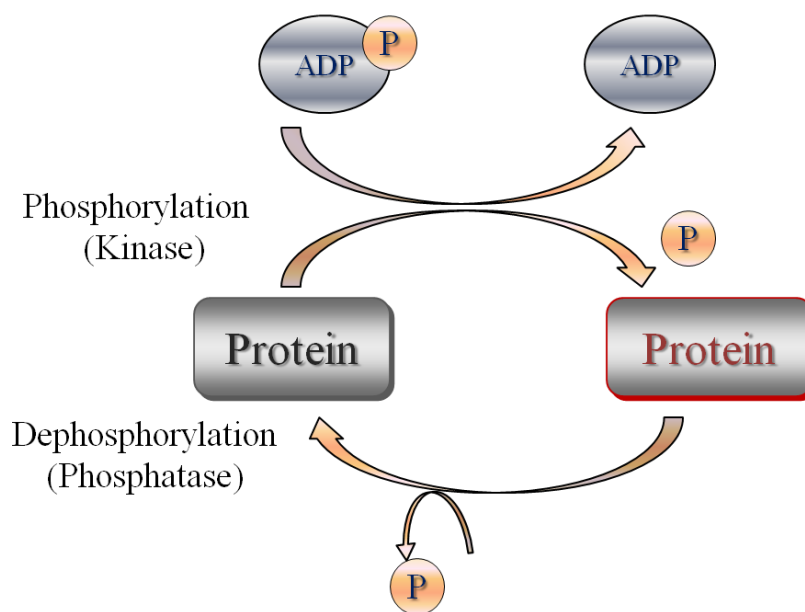


Figure 4.5. *Reversible protein phosphorylation*

In human cells the majority (86.4 %) of the proteins are phosphorylated on serine residues, while threonine and tyrosine share 11.8 % and 1.8%, respectively of the phosphorylated amino acids (Olsen et al., 2006). The covalent binding of phosphate groups to Ser/Thr/Tyr residues or their hydrolysis will result in the conformational change of the proteins regulating their activity. The level of phosphorylation at a particular protein site depends upon the balance between kinase and phosphatase activities (Hunter, 1995). If this balance is shifted towards either to phosphorylation or dephosphorylation, proteins may be under- or over-phosphorylated resulting in the dysfunction of the proteins.

4.5. Classification of protein phosphatases

The fully sequenced human genome contains 518 protein kinases (Johnson and Hunter, 2005) which can be classified into two families. Approximately 90 kinases are specific for Tyr residues (protein tyrosine kinases-PTK) and 428 kinases phosphorylate Ser/Thr residues (Brautigan, 2013). Reversible phosphorylation would suggest approximately the same numbers of protein phosphatases in the human genome. Interestingly, only 107 phospho-Tyr phosphatases (PTP) (Alonso et al., 2004) and approximately 40 phospho-Ser/Thr phosphatases were identified. The significantly lower number of protein Ser/Thr phosphatases (PP) can be explained by their diverse biochemistry. The action of Ser/Thr phosphatase (except PP2C enzymes which do not have additional regulatory subunits) is mediated by several regulatory subunits and interacting proteins, that bind the catalytic subunit and target the holoenzyme to specific subcellular locations and substrates, furthermore control its activity (Moorhead et al., 2007).

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 (Barford et al., 1995; Jackson and Denu, 2001; Pot and Dixon, 1992).

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) (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1980). 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 Ca^{2+} /calmodulin dependent phosphatase, also known as calcineurin. PP2C was found to be Mg^{2+} dependent, which was later identified by sequence analysis, as a different superfamily: metal ion dependent protein phosphatase, PPM (Cohen, 1997).

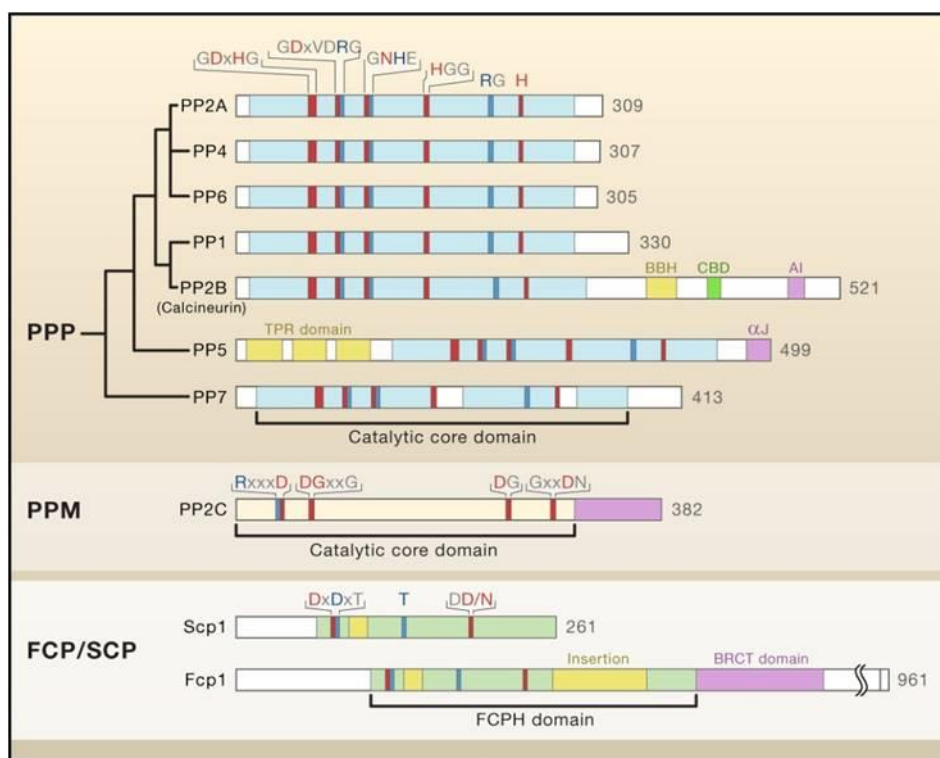


Figure 4.6. The three families of protein Ser/Thr phosphatases (Shi, 2009).

According to a new classification, Ser/Thr specific phosphatases fall into three families based on their sequences and their catalytic mechanisms. These are the phosphoprotein phosphatases (PPP), metal- ion dependent phosphatases (PPM) and FCP/SCP aspartate phosphatases (Csontos et al., 2007) (Brautigan, 2013) (Figure 4.6).

Members of PPP family involve PP1, PP2A, PP2B (calcineurin) and novel- type phosphatases, PP4, PP5, PP6, and PP7 (Figure 4.6). PP2C represents the PPM family, its activity is dependent on Mg^{2+} ions. The FCP/SCP (aspartate) enzymes are specific for YSPTSPS sequence repeat in the C-terminal domain of RNA polymerase II (Yeo and Lin, 2007). PP1 and PP2A are the most abundant phosphatases and they are involved in many cellular functions.

4.6. 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 (Cohen, 2002). This phosphatase is highly conserved (~70 %) in mammals during evolution. PP1 consists of a catalytic subunit and a regulatory subunit (R subunit). The catalytic subunit comprises three isoforms: PP1 α , PP1 β/δ , and PP1 γ . The latter one has two splice variants, the $\gamma 1$ and $\gamma 2$ (Moorhead et al., 2007). PP1 has several interacting protein partners that function as targeting subunits, substrates and/or inhibitors. Up to now more than 100 putative R subunits of PP1 have been identified (Cohen, 2002; Moorhead et al., 2007). These PP1 binding subunits share no common features, except the short conserved PP1c binding motif RVxF/W (R, arginine; V, valine; x, any amino acid; F, phenylalanine; W, tryptophan).

4.6.1. Myosin Phosphatase (MP)

One of the major factors regulating EC barrier function is the actomyosin-driven contraction. 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 (Garcia et al., 1995). There are two key enzymes involved in MLC phosphorylation level regulation: Ca^{2+} /calmodulin dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MP). The expression of MLCK was shown in many tissues, including the endothelium (Garcia et al., 1997a). As it was mentioned in section 4.2.4., and shown on Figure 4.3. the role of MP is crucial in the regulation of endothelial barrier function. MP is a type-1 protein phosphatase, composed of the PP1 catalytic

subunit (PP1 C β , initially described as PP1 C δ 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) (Hartshorne et al., 1998; Ito et al., 2004). The exact function of M20 is not identified yet. Hirano and coworkers were the first to reveal the expression of the MYPT subunit of MP in porcine aortic endothelial cells (Hirano et al., 1999). The direct involvement of PP1 in the regulation of endothelial barrier function and gap formation was shown by several laboratories (Diwan et al., 1997; Verin et al., 1995).

4.6.2. 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. Other possibilities are the dissociation or re-association of the holoenzyme, or binding of inhibitor proteins leading to the loss of activity of MP. The activation mechanisms are less explored, although it is known that cyclic nucleotides (cAMP and cGMP) are involved in it (Wu et al., 1996). Inhibition of MP by phosphorylation involves G- protein coupled receptors. Small GTPase RhoA and its downstream target, Rho kinase are involved in phosphorylating MYPT on its inhibitory sites: Thr696 and Thr853 (Somlyo and Somlyo, 2003). In addition, ZIP kinase (zipper-interacting protein kinase) was identified as an M20 regulator. It initiates MLC phosphorylation and leads to Ca²⁺ independent cell contraction (Niir and Ikebe, 2001). The integrin associated kinase (ILK) was also shown to inhibit MP activity by phosphorylating its inhibitory sites (Muranyi et al., 2002). Rho kinase and ILK are also implicated in the phosphorylation of MLC on Ser19 and Thr18, therefore directly controlling Ca²⁺ independent contraction.

The existence of specific inhibitory proteins such as CPI-17 (PKC potentiated inhibitory protein of 17 kDa), PHI-1, PHI-2 (phosphatase holoenzyme inhibitor 1 and 2), and KEPI (kinase-enhanced PP1 inhibitor) further increased the complexity of MP regulation (Cohen, 2002; Eto et al., 1999; Liu et al., 2002). CPI-17 blocks not only the catalytic subunit of PP1, it is able to inhibit the MP holoenzyme without dissociating its subunits, contrary to other inhibitors. 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 (Dubois et al., 2003; Watanabe et al., 2001). CPI-17 is well conserved in mammals (~80%); especially in the N-terminal residues 1–67 (coded by exon1). Phosphorylation of CPI-17 at Thr38 by PKC increased its inhibitory potency toward MP ~1000-fold (Eto et al., 1995; Kitazawa et al., 2000; Pang et al.,

2005). 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 (Kolosova et al., 2004). It was found that histamine and in a much lesser extent thrombin could cause CPI-17 phosphorylation. It was also revealed that CPI-17 can be phosphorylated by other kinases such as Rho kinase, however the contribution of PKC is more significant (Kolosova et al., 2004). Importantly, the activity of CPI-17 is controlled by PP2A that initiates its dephosphorylation (Takizawa et al., 2002) suggesting a novel possibility in MP regulation.

4.7. Protein Phosphatase 2A

4.7.1. Structural features of PP2A

PP2A is a ubiquitously expressed Ser/Thr phosphatase that makes up 1% of all cellular proteins and along with protein phosphatase 1 (PP1) accounts for over 90% of total Ser/Thr phosphatase activity in the cell (Depaoli-Roach et al., 1994). Several holoenzyme complexes of PP2A have been identified, isolated and characterized from a variety of tissues and cell types (Janssens and Goris, 2001). PP2A exists in two forms in the cells: the heterodimer core enzyme and the heterotrimer holoenzyme. The core dimer (PP2A_D) 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 (Figure 4.7).

PP2A C subunit has two distinct isoforms, C α and C β sharing 97% sequence identity (Stone et al., 1987). The presence or lack of associating subunits can alter PP2A activity. Two different genes (α and β) encode the PP2A A subunit. The majority of the enzymes contain PP2A A α isoform and a small fraction stands for PP2A A β . These two isoforms share 87% sequence homology (Hemmings et al., 1990). The regulatory B subunit has four unrelated families (B, B', B'', B''') encoded by multiple genes, with multiple splice variants creating a huge diversity of regulatory subunits and isoforms.

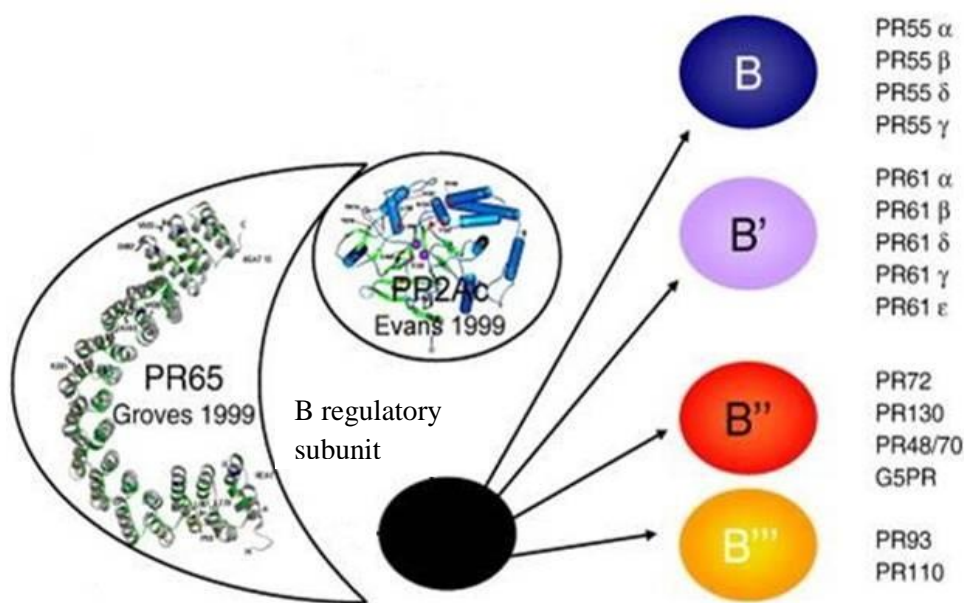


Figure. 4.7. Schematic overview of PP2A holoenzyme composition showing the families of the regulatory B subunits (modified picture of (Eichhorn et al., 2009))

In case of the B family numerous different nomenclatures are present in the literature. B (PR55, B55) has four isoforms: α , β , γ , or δ . B' is named as PR61, B56, or RTS1 (α , β , $\gamma^{1-3}\delta^{1-3}\epsilon$ isoforms) (Csortos et al., 1996; McCright and Virshup, 1995; Tehrani et al., 1996). B'' (PR48/PR72/PR130) also has several isoforms ($\alpha^{1,2}$, β , γ) (Hendrix et al., 1993a) and the B''' is also known as PR93/PR110. In mammalian cells there is a pool of PP2A AC dimer and as well as heterotrimeric forms of PP2A (Kremmer et al., 1997). Biochemical and genetic studies revealed that the regulatory B subunits are responsible for targeting the PP2A_D to their substrates and for the subcellular localization of the enzyme (Janssens and Goris, 2001; Janssens et al., 2005). Structural findings indicate that the highly conserved (~70% sequence homology among different species) C terminal six amino acids (TPDYFL) in the PP2A catalytic subunit have an important role in holoenzyme assembly and in the binding of distinct B subunits (Xu et al., 2008). The A subunit forms an elongated horseshoe - shape fold, which binds the catalytic C and the regulatory B subunits (Figure 4.7). The N-terminal repeats 1–10 of the A subunit are responsible for binding the B subunit, while the carboxy-terminal HEAT repeats 11–15 bind to the C subunit (Zhou and Kostic, 1993). The crystal structure of the heterotrimer PP2A holoenzyme also confirmed that the regulatory B subunit interacts with the 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 (Kowluru and Matti, 2012).

4.7.2. 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. For example, some viral proteins make complexes with PP2A changing its activity and substrate specificity (Kamibayashi et al., 1994). Posttranslational modifications like methylation or phosphorylation can be another possibility to regulate PP2A activity. The catalytic subunit undergoes reversible carboxyl-methylation and -demethylation at its C-terminal Leu309 catalyzed by PP2A-methyltransferase (PMT) and PP2A methylesterase (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. For example, carboxy-methylation was shown to enhance holoenzyme assembly, catalytic activation and association of PP2A C with phospho protein substrates (Bryant et al., 1999; Gentry et al., 2005; Longin et al., 2008). Other groups found that the activity of PP2A was largely unaffected by the addition of PME-1, and the regulatory subunit (PR55/B) could bind demethylated PP2A_D (Ikehara et al., 2007; Xing et al., 2006; Xu et al., 2008). Besides carboxy-methylation, PP2A was also shown to be affected by tyrosine kinases (pp60^{v-src}, pp56^{lck}), epidermal growth factor and insulin receptors which have been shown to inactivate PP2A (Chen et al., 1992). The activity of PP2A can also be regulated by toxins such as okadaic acid, microcystin or calyculin A (Sontag, 2001). More recently a phosphate ester antibiotic, fostriecin, also turned out to be a strong and relatively specific inhibitor of PP2A (Cheng et al., 1998).

The structural A subunit

The A subunit is the structural core of the PP2A holoenzyme tightly associated with the catalytic subunit forming a binding site for the regulatory B subunit. The scaffolding A subunit structure is entirely composed of 15 repeats of HEAT (huntingtin/elongation/A subunit/TOR (target of rapamycin)) motif, built from 39 amino acids. HEAT repeats are highly conserved sequences composed of two α -helices with an interhelical loop. Two alternative genes encode the two isoforms of PP2A A. A α and A β share high homology, although due to the unique structure of A β it is unable to substitute for A α in knockout mice (Hendrix et al., 1993b). Sequencing human tumor samples revealed that A β was mutated in primary lung tumors and lung tumor derived cell lines (Wang et al., 1998). Mutant PP2A A β could not form a

holoenzyme with the catalytic and the regulatory subunits. Interestingly, the expression level of A β was significantly reduced in the 50% of the tested 34 tumor cell lines, though the expression of A α was not reduced in these cells (Zhou et al., 2003). Mutations were described at the Glu64 residue of A α , which resides at the binding surface of B and C subunit, forming a hydrogen bond between A α and Leu309 amino acid chain of the catalytic subunit (Xu et al., 2006). The structural subunit can also associate with other proteins and is involved in special signaling pathways (Janssens and Goris, 2001; Ruediger et al., 1992).

The regulatory B subunit

The variable B subunit family has four different subfamilies that are called 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 α C holoenzyme is the most abundant and ubiquitous PP2A holoenzyme, and B α (B55) is the most predominant regulatory subunit of PP2A (Sontag, 2001). B α consists of at least 6 members coded by four genes with more variants likely to exist (Mayer et al., 1991). Several publications mention its involvement in cellular functions, and distinct subcellular localizations. For instance, B α associates with microtubules, neurofilaments, intermediate filaments (vimentin) as well as cytoplasmic, membrane and nuclear proteins (Sontag et al., 1995; Strack et al., 1998; Turowski et al., 1995; Turowski et al., 1999). The B family members are highly conserved, B α contains four to seven degenerate WD40 repeats (the number depends on the isoform), which reveals a β propeller structure in which four to seven antiparallel β strands are radially arranged around the center (Wall et al., 1995) (Figure 4.8). WD repeats are minimally conserved sequences. They comprise approximately 40 amino acids that typically end in tryptophan-aspartate (WD) and are thought to be responsible for protein-protein interactions (Neer et al., 1994). In addition to the β propeller arrangement, B α also contains two β hairpins and two α helices. These structural elements contribute to the formation of the acidic substrate-binding groove (Xu et al., 2008). Mutations in the acidic groove of B55 prevents the dephosphorylation of tau, a major substrate of PP2A B α in the brain (Xu et al., 2008).

The B55 α subunit makes extensive contacts with the A scaffolding subunit but little contact with the C catalytic subunit. The different B subunits could compete for interaction with the PP2A dimer and they are able to replace each other. *In vitro* data shows that B55 α can replace B55 β but not B', while B' can exchange both B55 α and B55 β (Mumby and Walter, 1993). The expression of small DNA tumor viruses depends on their tumor antigens and it is

well known that PP2A is a very important target for these viral antigens (Janssens and Goris, 2001; Sontag, 2001). Polyoma small T, middle T and SV40 small T can form stable complex with AC core dimer replacing B subunits and effectively comprise PP2A functions in transformed cells (Yang et al., 2005).

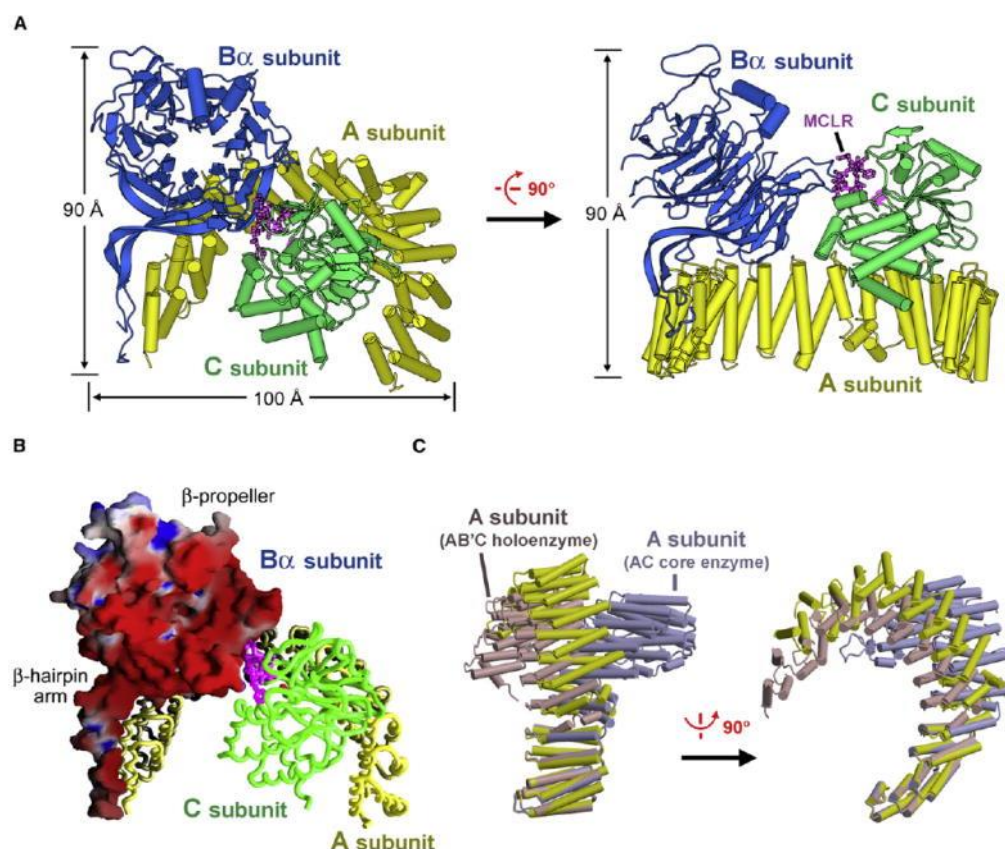


Figure 4.8. Overall structure of the heterotrimeric PP2A holoenzyme involving the B α subunit (Xu et al., 2008)

The B' family has eight members from five genes (McCright et al., 1996). The numerous isoforms' central region is highly conserved (~80%), although the C and N terminals are different. B' subunits show diverse tissue and subcellular localization involved in variable pathways (Csontos et al., 1996; Janssens and Goris, 2001). B'' family members PR72 and PR130 are encoded by the same gene, they differ in the N terminal region (Hendrix et al., 1993a). Member of B'' family, PR72, was shown to associate with the tumor suppressor retinoblastoma protein and mediate its dephosphorylation (Magenta et al., 2008). Overall, B subunits have been shown to be implicated in many cellular functions, however, their role in EC barrier function needs to be uncovered.

4.7.3. Biological functions of PP2A

PP2A has an important role in the control of cell cycle, transformation, growth or apoptosis, because it is involved in several signaling pathways. PP2A was first suggested to act as a tumor suppressor, when its specific inhibitor okadaic acid applied on mice skin resulted in tumor formation and the inhibition of PP2A activated several tumor promoting pathways (Suganuma et al., 1988). As it was mentioned in the previous paragraph, tumor promoting viruses can affect PP2A activity by replacing the B regulatory subunits in the holoenzyme. Alteration of PP2A by viral proteins led to the downregulation of similar pathways which found to be disturbed by okadaic acid (Eichhorn et al., 2009). Both polyoma small T and middle T antigens could induce transformation in cells, which could be achieved by the inhibition of PP2A. The overexpression of small T antigen causes a decrease in B55 level, resulting in hyperphosphorylation of proteins which are involved in cell growth (Zhou et al., 2003).

Another major function of PP2A is to control the mitogen activated protein (MAP) kinase pathway. PP2A may contribute both as a negative and as a positive regulator in this pathway in a tissue or a cell specific manner (Eichhorn et al., 2009). Growth factors induce the translocation of B55 to the plasma membrane where it associates with and dephosphorylates membrane bound kinase suppressor of Ras (KSR1) and Raf1, resulting in the downstream activation of MAP kinase pathway (Ory et al., 2003). It was also found in *Drosophila* and mammals that B55 γ interacts with and dephosphorylates c-src and negatively regulates the MAPK pathway (Eichhorn et al., 2007).

PP2A exerts inhibitory effects on Wnt/ β -catenin signaling that have a significant role in development and tumorigenesis (Moon, 2005). Multiple kinases are involved in β -catenin phosphorylation such as casein kinase I (CKI) and glycogen synthase kinase 3 β (GSK3 β) (Aberle et al., 1997; Rubinfeld et al., 1996) leading to the ubiquitination of β -catenin. PP2A B α was shown to directly interact with β -catenin and it regulates β -catenin dephosphorylation in Wnt-signaling in colon carcinoma cell lines (Zhang et al., 2009) (mentioned in 4.3.). β -catenin also associates with axin, GSK3- β and APC (adenomatous polyposis coli). The latter one was shown to bind PP2A B subunits, PR61 α and PR61 δ by using bacterial two hybrid screening (Seeling et al., 1999). PP2A can stabilize β -catenin directly. PP2A has been reported to form a complex with E-cadherin and β -catenin at the plasma membrane resulting in the stabilization of both proteins. Collectively these observations highlight the role of PP2A in many biological,

cellular functions and in diverse signaling cascades. Undoubtedly further studies will lead to a more complete understanding of the biological functions of PP2A.

4.7.4. PP2A and the cytoskeleton

Endothelial barrier function is regulated by the precise balance between adhesive cell-cell and cell-matrix forces and actomyosin contractile forces imposed by the endothelial cytoskeleton. This complex network comprises actin microfilaments, microtubules (MT) and intermediate filaments (IF), regulating cell shape and dynamics. Paracellular gap formation evoked by barrier disruptive agents for example thrombin or histamine, results in increased endothelial permeability and is governed by the coordinated communication among cytoskeletal elements. Reversible phosphorylation of cytoskeletal or cytoskeleton associated proteins is critical in the maintenance of endothelial barrier integrity (Dudek and Garcia, 2001). In this process the role of PP1 has been well established, while the involvement of PP2A activity is less studied.

The cytoskeletal substrates of PP2A and their exact function in cell contraction or relaxation are not well characterized, though some recent literature data confirms its involvement in the regulation of cytoskeleton associated proteins. For example Takizawa and coworkers examined the role of phosphatases in the dephosphorylation of MP inhibitor, CPI-17. They found that PP2A catalyzes the dephosphorylation and inactivation of CPI-17 in smooth muscle cells, thus prevents cell contraction (Takizawa et al., 2002). Another important finding claims that the specific inhibition of PP2A by okadaic acid is crucial in the phosphorylation and translocation of MYPT1 in HepG2 cells (Lontay et al., 2005). These data demonstrate the cross-talk between MP and PP2A in the cytoskeleton organization.

Actin filaments are dynamic structures with critical importance to EC permeability. These cytoskeletal structures are controlled by various actin binding proteins, for instance plectin, spectrin, gelsolin, caldesmon, cofilin, HSP27 (small heat shock protein 27) which are important in actin polymerization/depolymerization. Cofilin, an actin-depolymerizing protein, is essential for the functional dynamics of the actin cytoskeleton and for cell viability, is dephosphorylated by PP2A in human T lymphocytes (Ambach et al., 2000). *In vitro* experiments revealed that purified PP2A dephosphorylated HSP27. These data were further reinforced when human lung fibroblasts were treated with phosphatase inhibitors and HSP27 phosphorylation was affected by okadaic acid and calyculin A or cantharidin (PP2A inhibitor), but not the PP2B inhibitor, cyclosporin A (Cairns et al., 1994). Furthermore, caldesmon

phosphatase was identified as PP2A in chicken gizzard smooth muscle (Pato et al., 1993). The regulation of intermediate filaments is mostly unexplored in the endothelium, although there are some literature data about the involvement of PP2A in the dephosphorylation of IF proteins (Nakamura et al., 2000; Turowski et al., 1999).

There is a growing body of evidence, showing the importance of PP2A activity in MT stabilization (Hiraga and Tamura, 2000; Sontag et al., 1995; Sontag et al., 1999). Several literature data found the association of PP2A with microtubules and MT-associated proteins (MAPs), for example tau. A β C holoenzyme is responsible for the dephosphorylation of brain tau and might be involved in the pathophysiology of Alzheimer's disease (Sontag et al., 1999). Similarly, our group revealed a substantial amount of PP2A associating with HSP27 and tau in MT-enriched fraction of pulmonary artery ECs (Tar et al., 2004). Previous results of our group further support the significance of PP2A in the vascular endothelium. 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 thrombin- or nocodazole-induced EC barrier dysfunction (Tar et al., 2006). All the above mentioned data highlight the importance of PP2A in the endothelial cytoskeleton, and claims the significance of microfilaments and microtubules in EC barrier regulation, although there remains a lot to explore.

5. AIMS

Published data of our group demonstrated that the over-expression of the structural (PP2A A) and the catalytic (PP2A C) subunits of PP2A attenuates barrier-disruptive effect of edemagenic agonists on EC monolayer (Tar et al., 2006). The PP2A core enzyme is targeted by the regulatory B subunits to different subcellular localizations, and determines the substrate specificity of the enzyme, although the regulatory role of B subunit in cytoskeleton organization is still unknown.

Literature data demonstrated that the inhibition or the lack of Ser/Thr phosphatases cause hyperphosphorylation of β -catenin on Ser/Thr residues and resulted in the loss of cell-cell contacts (Serres et al., 1997). Another published work claims that B α can regulate PP2A-mediated β -catenin dephosphorylation during Wnt-signaling in *Drosophila* (Zhang et al., 2009). Based on these findings we hypothesized that PP2A phosphatase can be intimately involved in β -catenin signaling and it may control the assembly of cell-cell contacts in endothelial cells.

Phosphorylated CPI-17 is a potent inhibitor of MP which binds to MP catalytic subunit making the phosphatase unable to dephosphorylate its primary target, MLC, therefore leading to cell contraction, and endothelial barrier dysfunction (Eto et al., 1995; Verin et al., 1995). PP2A can be potentially implicated in MLC phosphatase (MP) activation via dephosphorylation of CPI-17 (Hersch et al., 2004; Ito et al., 2004) and thus opposing contraction and permeability increase.

Until now limited information is available regarding the role of dephosphorylation of cytoskeleton associated and adherens junction proteins in EC barrier regulation. Our goal is to further examine novel signaling pathways involved in EC barrier preservation, focusing on the link between PP2A activity, dephosphorylation of putative PP2A cytoskeletal targets and CPI-17.

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 α .
- 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).

6. MATERIALS AND METHODS

6.1. Materials

6.1.1. Reagents and chemicals

Reagents and chemicals were purchased from the companies as listed below.

Abcam: Gelsolin mouse mAb, Amersham Biosciences: Hybond ECL nitrocellulose membrane, Applied Biophysics: ECIS chambers, Applied Biosystems: TRIZOL, Bio-Rad: pre-stained protein standard, agarose, Cell Signaling Technology: MYPT rabbit pAb, Plectin-1 rabbit pAb, PP2A_B rabbit mAb, β -catenin rabbit mAb, phospho- β -catenin (S552) rabbit mAb, VE-cadherin rabbit mAb, Lumiglo ECL reagent, Corning Cellgro: Trypsin EDTA (1X and 10X), PBS, Dharmacon: PP2A_{B α} silencing RNA, non-targeting silencing RNA, DharmaFECT1 transfection reagent, EMD Biosciences: Protease Inhibitor Cocktail Set III, PMA, GE Healthcare: protein G sepharose Invitrogen: V5 mouse mAb, MEM, DMEM, Lonza: EGM-2-MV medium and bullet kit, Millipore: β -tubulin mouse mAb, CPI-17 and phospho-CPI-17 (Thr38) rabbit pAb, Plakoglobin mouse mAb, p120 Catenin mouse mAb, PVDF membrane, Molecular Probes: goat anti-mouse Alexa-488 or -594, goat anti-rabbit Alexa-488 or Alexa-594-conjugated antibodies, Texas Red-conjugated phalloidin, ProLong Gold Antifade medium, Promega: M-MLV reverse transcriptase, GoTaq® Flexi DNA Polymerase, Roche: XtremeGENE transfection reagent Santa Cruz: c-myc rabbit or mouse pAb, spectrin α II mouse mAb, Sigma-Aldrich: β -catenin mouse mAb, Actin mouse pAb, Anti-V5 Agarose Affinity Gel antibody produced in mouse, Anti-c-Myc Agarose Affinity Gel antibody produced in rabbit, bovine and human thrombin, Tocris Bioscience: okadaic acid, fostriecin
All other chemicals were from Sigma Aldrich.

6.1.2. Culture media

Complete MEM: 90% (v/v) MEM, 1% antibiotic and antimycotic solution, 1% sodium-pyruvate, 10% FBS, 1% non-essential amino acids

EGM-2 MV BulletKit: Basal EGM-2 (Endothelial Growth Medium) supplemented with 5% FBS SingleQuots Kit (Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic acid, hEGF, GA-1000)

Complete DMEM: (Dulbecco's Modified Eagle Medium) (Gibco): 90% (v/v) DMEM, with 2 mM glutamine, 10% FBS and 1% antibiotic-antimycotic

Antibiotic and Antimycotic Solution (Gibco): 10,000 units/ml of penicillin, 10 mg/ml of streptomycin, and 25 µg/ml of Fungizone

Fetal Bovine Serum (FBS) (Gibco)

6.1.3. Buffers and solutions

10x PBS (pH 7.4): 14.4 g/l Na₂HPO₄, 2.4 g/l KH₂PO₄, 2 g/l KCl, 80 g/l NaCl

10x TBS (pH 7.4): 25 mM Tris, 150 mM NaCl, 2 mM KCl,

10x TBST (pH 7.4): TBS + 0,1 % Tween 20

1x TE puffer (pH 8.0): 10 mM Tris-HCl, 1 mM EDTA,

1x TAE puffer (pH 8.0): 4 mM Tris-HCl, 0.1 mM EDTA, 0.114% acetic acid

5x SDS-sample buffer: 50% glycerin, 10% SDS, 310 mM Tris-HCl, 100 mM DTT, 0.01 % bromphenol blue

DNA loading buffer (6X): 66.6% sucrose, 0.416 % bromophenol blue

Running Buffer (pH 8.3): 25 mM Tris, 192 mM glycine, 0.1% SDS

Transfer buffer: 120 mM Tris-HCl, 40 mM glycin, 20 v/v% methanol

Lysis buffer: 10 mM Tris-HCl pH 7.5; 140 mM NaCl; 1% Triton-X-100; protease inhibitor cocktail (1:200); 10 mM EDTA; 0.1% SDS

IP buffer: 20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 150 mM NaCl, 3 mM EDTA, 3mM EGTA, protease inhibitor cocktail (1:200)

6.1.4. PCR primers

Primer	Accession Number	Sequence (5' to 3')	T _m (°C)	Expected product size (bp)
PP2A partial coding seq.	NM_002717			
B α forward		CAG CAC CTT CCA GAG CCA	59.25	559
B α reverse		GGC AGA TGC CCT CAT GTC	57.85	
PP2A Full length seq.	NM_002717			
B α forward		ATG GCA GGA GCT GGA G	55.01	1341
B α reverse		ATT CAC TTT GTC TTG AAA TAT ATA CAG	54.43	

Table 1. PP2A B α primers for PCR

6.2. Methods

6.2.1. Cell cultures and treatments

Our experiments involved human and bovine macrovascular endothelial cells (HPAEC and BPAEC, culture line-CCL209) which were from American Type Tissue Culture Collection (Rockville, MD) and human microvascular endothelial cells (HLMVEC) obtained from Lonza Group Ltd. (Walkersville, MD). Human Embryonic Kidney 293T (HEK 293T) cells were from European Collection of Cell Cultures (Salisbury, UK). All cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

In our experiments the BPAE cells were maintained in complete MEM, HLMVE and HPAE cells were cultured in EGM-2 MV completed with supplements and growth factors. Complete DMEM was used to maintain HEK cells. Cells were cultured in T75 flasks. After reaching 100% confluence cells were subcultured. The spent cell culture media was removed and discarded from the culture vessel. Cells were gently washed using PBS without calcium

and magnesium. Then pre-warmed 1x Trypsin-EDTA was added on the cells and incubated for 2 minutes. Cells were observed under the microscope for detachment. When cells have detached, complete culture media (8 ml) was applied to the cells. Cell suspension was transferred to 50 ml Falcon tube, and 5 ml of complete media was added into the flask to collect all remaining cells from the culture vessel. Cell suspensions were diluted according to the recommended seeding density of each cell line. The appropriate volume of cell suspension was pipetted into new T75 flask, and returned to the humidified 5% CO₂ incubator. BPAEC was utilized at 16-23 passages, HPAEC and HLMVEC was used at passages 4-8.

Before any stimulation, cells were grown to 80-90% confluence. Macrovascular cells were treated with 5 nM okadaic acid (90 min), 100 nM fostriecin (90 min), or 50 nM thrombin (30 min). Microvascular cells were treated similarly except lower (0.5 nM) thrombin concentration. After stimulation cells were fixed on glass coverslips with 3.7% formaldehyde solution for immunofluorescent staining. For Western blot experiments cells were scraped in lysis buffer, centrifuged, and then boiled in the presence of 1x final concentration of SDS sample buffer.

6.2.2. Immunofluorescent staining

Cultured microvascular or macrovascular endothelial cells grown on 0.2% gelatin coated glass coverslips were fixed with 3.7% formaldehyde solution in TBS for 10 minutes and washed three times with TBS. The cells were permeabilized with 0.2% Triton X-100 in TBS supplemented with 0.1% Tween 20 (TBST) for 5 min, washed three times with TBS, and blocked with 2% BSA in TBST for 1 hour. Incubation with specific primary antibody, in 1:100 dilutions with blocking solution, was performed for 1 hour at room temperature. After three washes with TBS, cells were incubated with appropriate secondary antibody (applied in 1:300 dilution) conjugated with fluorescent dye Alexa-488 (green) or Alexa-594 (red) (Table 2.) for 1 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.

Secondary antibody	Absorption wavelength (nm)	Emission wavelength (nm)
Alexa Fluor 488	496	519
Alexa Fluor 594	590	617
Texas Red Phalloidin	591	608

Table 2. Properties of the fluorescence dye conjugated secondary antibodies.

6.2.3. Immunofluorescent microscopy

After immunostaining, the glass slides were observed using a Nikon video-imaging system (Nikon Instech) consisting of a phase-contrast inverted microscope Nikon Eclipse TE2000 connected to digital camera and image processor (Hamamatsu Photonics) 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 Adobe PhotoShopCS5 Imaging software.

6.2.4. Image analysis of stress fiber formation

Texas Red-stained EC monolayers treated with either thrombin or PP2A B α siRNA were observed by a Zeiss Axiolab microscope (FluoView program) using 63 x oil immersion objective. 8-bit images were analyzed using Image J 1.46R. For evaluation 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 Sigma GraphPad Prism 5 software (GraphPad Software, San Diego, CA)

6.2.5. 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 (Kolosova et al.,2004) constructs were prepared in Dr. Verin's laboratory. The PP2A B α pcDNA 3.1 V5/His construct was created as it is described in 6.2.9.

HLMV (CPI-17 expression) cells were grown on glass coverslips in 12 well plates or 10 cm cell culture dishes until cells reached 80-90% confluence. Then cells were incubated with 1 ml or 10 ml of OPTI-MEM medium containing 1 μ g or 10 μ g DNA and 3 μ l or 30 μ l of X-tremeGENE HP transfection reagent for 6 hours in CO₂ incubator at 37°C. After washing (EGM-2-MV +5% FBS), cells were incubated for an additional 24-48 hours and used for immunoprecipitation experiments.

HEK (PP2A B α , inhibitor-2, HSP27 expression) cells were grown in 10 cm cell culture dishes and were transfected at 80-90% confluence using 10 μ g DNA: 20 μ l PEI ratio. After 6 hours of transfection cells were washed (DMEM+10% FBS), incubated for an additional 24-48 hours, then used for pull down experiments.

6.2.6. *In vitro* pull-down assay

Transfected HEK cells were rinsed with ice cold PBS, scraped and lysed in lysis buffer, followed by 10 minutes centrifugation (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 α , HSP27 or inhibitor 2. Next, the resin was centrifuged for 2 min at 8,200 g. Meanwhile, BPAEC were washed with ice cold PBS and scraped in lysis buffer (without any detergents) followed by sonication (3x 10 sec) and centrifugation (8,200 g, 4°C). The supernatant of EC cell lysate, was added onto the resin to which the recombinant B α , 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.

6.2.7. Immunoprecipitation

HLMVE cells were transfected with CPI-17 pcDNA myc/His construct using XtremeGene transfection reagent according to the manufacturer's instructions. After 72 hours of incubation, cells were washed three times with ice-cold PBS and lysed in IP buffer. The cell lysate was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was pre-cleared with protein G Sepharose at 4°C for 3 hours with gentle rotation to prevent nonspecific binding. The pre-cleared supernatant was incubated with EZ-view Red Anti c-myc Affinity gel. After overnight incubation at 4°C with gentle rotation, the resins were centrifuged. The supernatant was removed, and the remained beads were washed three times with IP buffer, resuspended with 1x SDS sample buffer, and then boiled for 5 min. The eluted samples were further analyzed by Western blot.

6.2.8. Depletion of endogenous PP2A B α in endothelial cells

To reduce the content of endogenous PP2A B α protein ECs (HLMVEC, HPAEC and BPAEC) were treated with SMART selection-designed PP2A B α -specific small interfering RNA (siRNA) duplex oligonucleotides (in 50 nM final concentration), which guide sequence-specific degradation of the homologous mRNA. Non-targeting (#1) siRNA duplexes (in 50 nM final concentration) were used as controls (non-siRNA). Cells were transfected at 70-80% confluence using DharmaFECT1 transfection reagent according to the manufacturer's protocol. 48 hours later, cells were used to measure transendothelial electrical resistance, for immunofluorescence staining or for Western blot analysis of siRNA-induced specific PP2A B α depletion.

6.2.9. RNA isolation and RT-PCR

Total RNA was isolated from endothelial cells using TRIZOL according to the manufacturers' instruction. Concentration and purity of RNA was determined by NanoDrop Spectrophotometer measuring absorbance at 260 and 280 nm. In the reverse transcription 2 µg of RNA was incubated in the presence of 0,111 µM oligo(DT) in a total volume of 15 µl. The mixture was heated to 70°C for 5 minutes then it was cooled immediately on ice. The following components were added to the template: 5 µl M-MLV 5X Reaction Buffer, 5 mM dNTPs, 25 units Recombinant RNasin® Ribonuclease Inhibitor, 200 units of M-MLV reverse transcriptase and nuclease-free water to a final volume of 25 µl. The reaction mixture was incubated for 60 minutes at 37 °C.

PCR reactions were performed using GoTaq® Flexi DNA Polymerase in a reaction mixture containing 10 µl 5X Green GoTaq® Flexi Buffer1, 1.5 µl 25 mM MgCl₂ solution, 0.5 µl 10 mM dNTP, 25-25 pmol reverse and forward primer, 1.25 U GoTaq® DNA Polymerase, 5 µl DNA template and nuclease-free water to 50 µl. The PCR protocol contained 3 stages, which were as follows: stage1: 1 cycle of denaturation at 94 °C for 1 min followed by stage 2: 30 cycles of denaturation at 94 °C for 30 sec, annealing at optimized temperature for each primer set based on the primer's T_m (see in Table 1.) for 30 sec and extension at 72 °C for 1 min, then stage 3: final extension 72 °C, 5 minutes, 1 cycle, hold 4 °C indefinite 1 cycle. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

Full-length PP2A Bα was first cloned into pCR2.1-TOPO, then subcloned into pcDNA3.1/V5-His using *EcoRI* restriction site.

6.2.10. 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 (Applied Biophysics, Troy, NY), as described previously (Garcia et al., 1997b). HPAEC and HLMVEC were plated on small gold microelectrodes (250 µm diameter) 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 of incubation TER was monitored in response to EC barrier disruptive agent (thrombin). Electrical resistance was measured dynamically across the endothelial monolayer and was determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion, and the resistance between cells (Figure 6.1). As cells grow on the microelectrode, the TER increases (maximal at

confluence), whereas cell retraction, rounding, or loss of adhesion is reflected by a decrease in TER (Giaever and Keese, 1993).

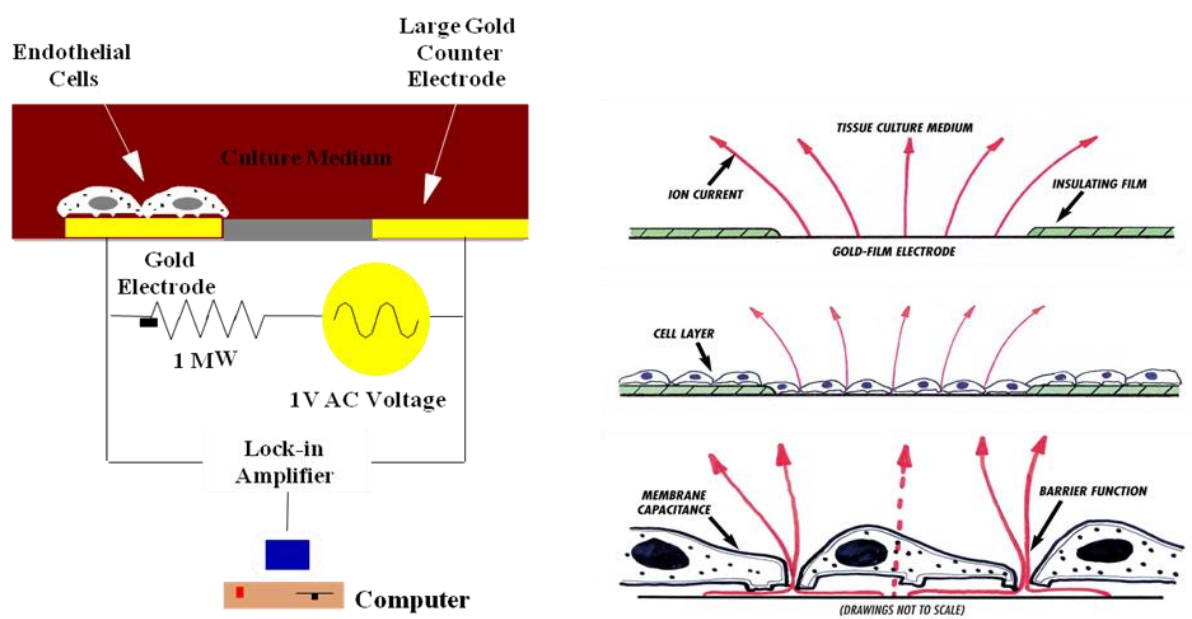


Figure 6.1. Method for assaying endothelial barrier properties in vitro with ECIS (Electrical Cell-substrate Resistance Sensing System)

Electrical resistance increased immediately after the cells attached to and covered the electrodes, and the resistance achieved a steady state when the cultured EC became confluent. Thus, experiments were conducted after the electrical resistance achieved a steady state. Resistance data were normalized to the initial (steady state) voltage and plotted as a normalized resistance.

6.2.11. Western blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to molecular mass, using the Bio-Rad Mini Protean equipment. To visualize the apparent molecular weights Bio-Rad Dual Color protein standard was used. After separation by electrophoresis (35 mA, 1-1,5 h), proteins were transferred to nitrocellulose or to PVDF membranes using an electric current (25 V for 16 h or 100 V for 90 min at 4 °C). The nonspecific binding sites of the membranes were blocked with incubation in 5% non-fat dry milk in TBS-Tween20 (TBST) for 1 hour and probed with the primary antibody of interest diluted 1:1000 and then with HRP-conjugated secondary antibody (1:5000). Between each step membranes were washed for 3x10 min with TBST. Immunoreactive proteins were detected on X-ray film

(*Agfa CP-BU New film*) with Lumiglo enhanced chemiluminescent detection system (ECL) reagents according to the manufacturer's directions.

6.2.12. Statistical analysis

All experiments were performed three or more times. AxioVision LE64, Image J 1.46R and Photoshop CS6 were applied to edit immunofluorescent pictures. The analysis of ECIS results was performed with Excel (Microsoft Corporation), and GraphPad Prism5 was used for graphical data presentation. To analyze Western blot results, films were digitized 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.

7. RESULTS

7.1. Role of PP2A in endothelial cell cytoskeleton organization

7.1.1. Characterization of okadaic acid and fostriecin induced F-actin reorganization

Human lung microvascular ECs and bovine pulmonary artery ECs were treated either with vehicle or with specific protein phosphatase 2A (PP2A) inhibitors: okadaic acid (OA) or fostriecin. Okadaic acid is an inhibitor of the Ser/Thr protein phosphatases PP1 and PP2A, exhibiting a much greater affinity towards PP2A ($IC_{50} = 15\text{-}20\text{ nM}$ for PP1 vs 0.1 nM for PP2A) (Wera and Hemmings, 1995). In our experiments cells were challenged with 5 nM OA for 90 minutes. Fostriecin is an antitumor agent and is an even more selective inhibitor of PP2A compared to PP1 ($IC_{50} = 131\text{ }\mu\text{M}$ for PP1 vs 3.2 nM for PP2A) (Swingle et al., 2007; Walsh et al., 1997). We used 100 nM of fostriecin treatment for 1 hour in our studies.

The specific inhibition of PP2A was accompanied by the formation of F-actin stress fibers and the partial dissolution of the peripheral microtubules (Figures 7.1 and 7.2). Texas Red-phalloidin staining indicated the alteration of actin cytoskeleton, we observed cortical F-actin dissolution and stress fiber formation reflecting a more contractile phenotype of cells in the monolayers (Figure 7.1B,D,F and Figure 7.2A,C). Besides the effect of PP2A inhibitors on microfilaments, tubulin staining revealed the partial depolymerization of the microtubules due to the treatments (Figure 7.1A,C,E and Figure 7.2B,D). Our results further support previous findings from our group that PP2A plays a crucial role in the regulation of endothelial cytoskeleton structure organization (Tar et al., 2006).

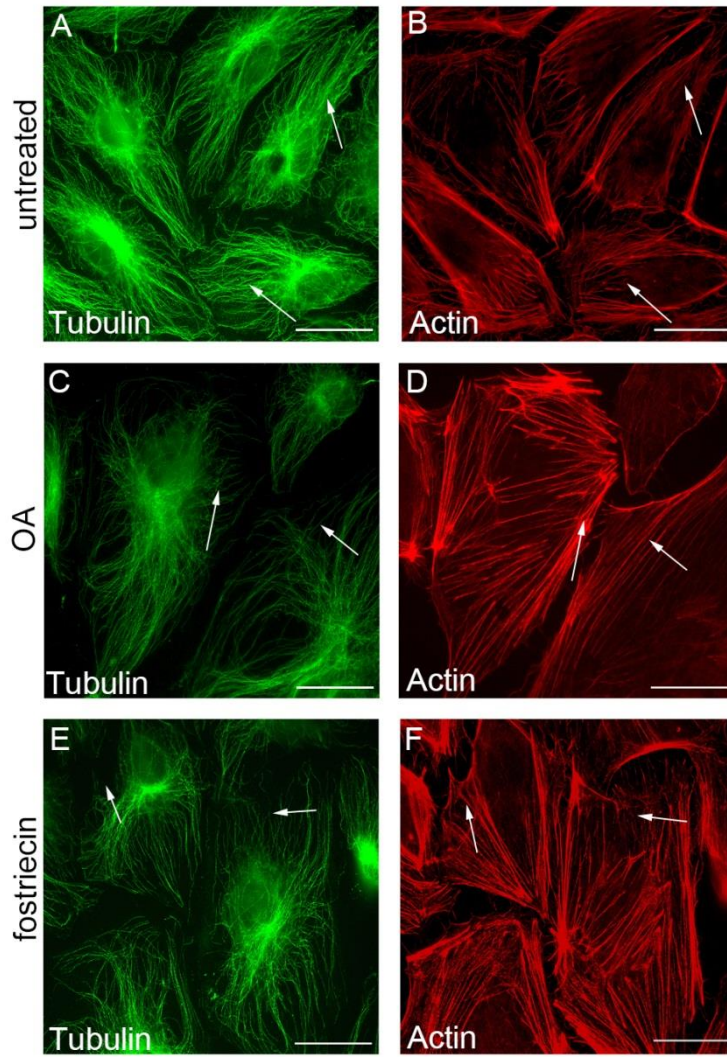


Figure 7.1. The lack of PP2A activity changes the cytoskeleton structure of microvascular endothelial cells. Confluent cultures of HLMV endothelial cells (A-F) were treated either with vehicle (A,B) (0.1 % DMSO), with 5 nM OA (C, D) for 90 min, or with 100 nM fostriecin (E, F) for 1 hour. Cells were double stained with anti- β - tubulin antibody (A, C, E) and with Texas Red- phalloidin (B, D, F) to visualize microtubules and microfilaments, respectively. Arrows are pointed to the normal actin or tubulin filaments (A,B) or to the actin stress fibers or depolymerized microtubules (C-F). Fluorescent images were captured with an Olympus Flouview FV1000 confocal microscope. Scale bars: 200 μ m. A and B, C and D, E and F are parallel images.

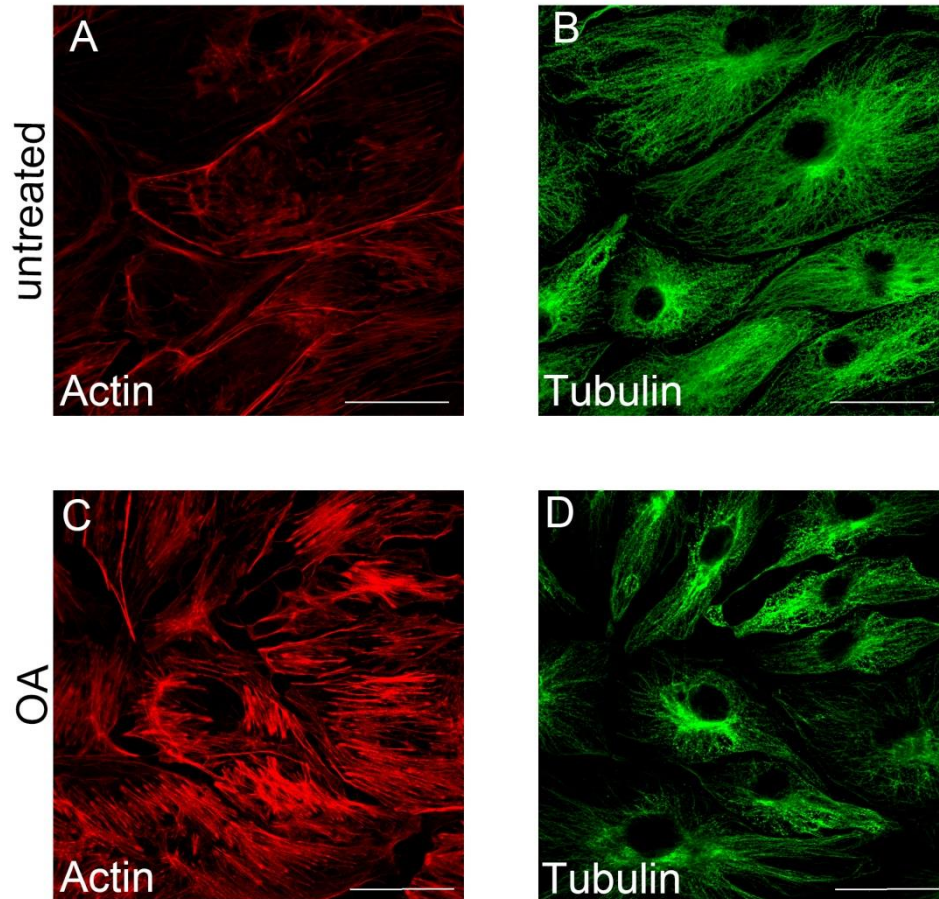


Figure 7.2. Inhibition of PP2A activity changes the cytoskeleton structure of macrovascular endothelial cells. BPAEC monolayers (A-D) were double stained as described for Figure 1 to visualize microfilaments (A,C) and microtubules (B,D) after treatment with vehicle (0.1 % DMSO) (A,B), or with 5 nM OA (C, D) for 90 min. Pictures were taken with an Olympus Flouview FV1000 confocal microscope. Scale bars: 200 μ m. A and B, C and D, are parallel images.

7.1.2. Depletion of PP2A B α in endothelial cells

The inhibition of PP2A activity caused significant changes in the endothelial cytoskeleton as we show it in Figures 7.1 and 7.2. We intended to further clarify the role of the most abundant holoenzyme form of PP2A, AB α C. To reduce the level of the endogenous B α subunit in EC we used small interfering RNA technique as described in the Material and Methods. We performed reverse transcription (RT) PCR (Figure 7.3A) to verify the efficiency of PP2A B α silencing at the mRNA level. To confirm our results on protein levels as well, we analyzed the transfected cells using Western blot (Figure 7.3B). Membranes were probed with a specific antibody against PP2A B and actin was also stained. Analysis of our results confirmed about 70-80% decrease in the endogenous level of PP2A B α compared to the non-silencing RNA transfected cells with both methods.

7.1.3. Effect of PP2A B α depletion on actin cytoskeleton remodeling

After demonstrating the efficiency of the depletion with Western blot and RT-PCR we further proved the silencing of the B subunit by immunofluorescent staining of PP2A B in EC monolayers transfected with PP2A B α specific siRNA or with non-siRNA (Figure 7.3Ca-b). We also intended to investigate the effect of the depletion of B α 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 (Tar et al., 2004). To examine the direct involvement of the B α regulatory subunit in agonist induced EC barrier regulation, we challenged the PP2A B α depleted cells and non-siRNA transfected cells with thrombin (50 nM, 30 min). Importantly, thrombin treatment further enhanced the effect of PP2A B α depletion on F-actin stress fiber formation (Figure 7.3Ce) compared to undepleted cells (Figure 7.3Cf). To further refine our results we conducted quantitative analysis of our pictures acquired by staining of actin microfilaments (Figure 7.3Cg). The images were analyzed using Image J software. For evaluation of stress fiber formation, actin fibers were marked and the ratio area covered by stress fibers to the whole cell area was determined. This analysis confirmed the increased stress fiber formation both in thrombin treated or in PP2A B α depleted cells.

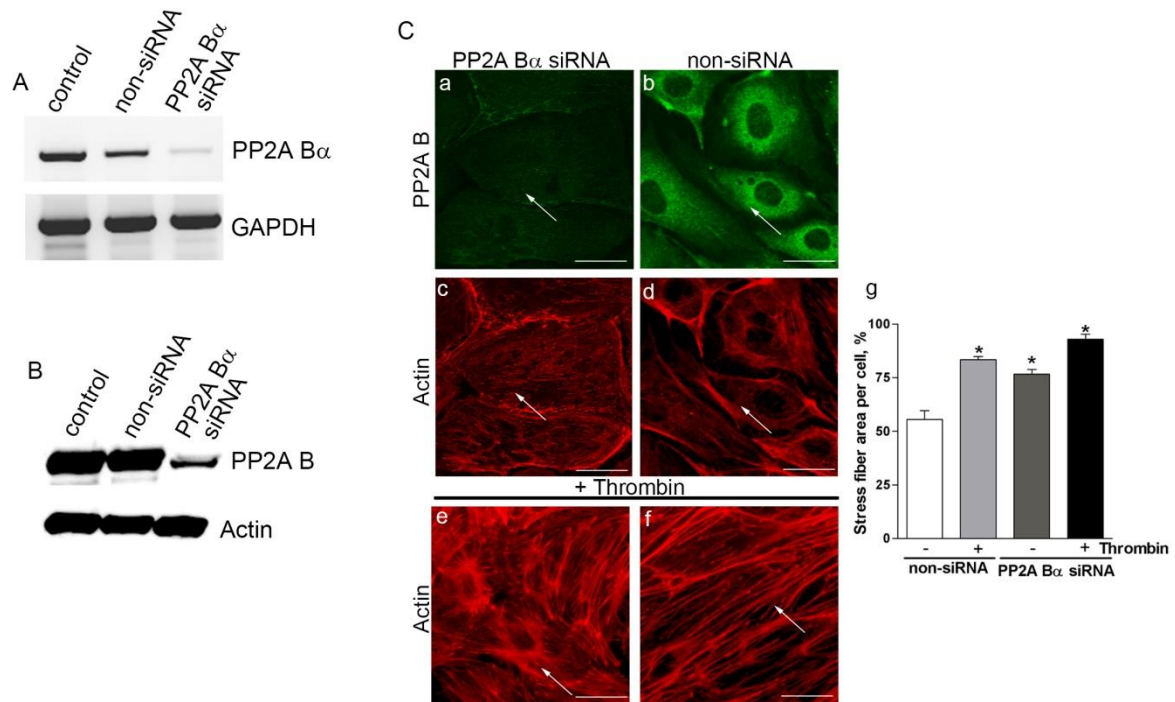


Figure 7.3. PP2A B α depletion and its effects on actin cytoskeleton. PP2A B α was depleted in BPAEC using specific siRNA as described in Materials and Methods. (A) BPAEC monolayers were transfected with PP2A B α specific siRNA and control (non-si) RNA. 72 hours later cells were scraped to isolate total RNA and to perform RT-PCR using specific B α primer pairs. GAPDH was used as an inner control. (B) BPAEC monolayers were transfected with PP2A B α specific siRNA and non-siRNA. 72 hours later cells were scraped in 2x SDS sample buffer, boiled and used for Western blot analysis. To detect the endogenous B α specific primary antibody was used against this protein. Actin was used as a loading control. (C) BPAEC monolayers were transfected with PP2A B α specific siRNA and non-siRNA. 72 hours later transfected cultures without (a-d) or with (e-f) thrombin treatment (50 nM, 30 min) were double-stained with specific primary antibody against PP2A B (a,b) and with Texas Red-phalloidin (c-f). Arrows highlight the actin filaments in control and treated cells. Pictures were taken with a Zeiss Axiolab microscope, scale bars: 200 μ m. (C/g) Quantitative analysis of stress fiber formation induced by thrombin was evaluated by morphometric analysis using Image J program as described in Materials and Methods. Measurements are presented as means \pm SEM, $n=14$. Changes considered significant are indicated by * ($P < 0.05$ vs non-si without thrombin), and # ($P < 0.05$ vs non-si with thrombin).

7.1.4. PP2A B α depletion significantly enhances thrombin induced EC barrier loss

As described in Birukova et al., (2004), measurements of TER across human pulmonary EC monolayer after thrombin stimulation demonstrated a significant decrease in transendothelial resistance reflecting a dramatic EC barrier compromise (Birukova et al., 2004a). Previous data from our group confirmed the involvement of PP2A in EC barrier protection against edemagenic agonists, thrombin and nocodazole (Tar et al., 2004). In order to directly examine the role of PP2A B α subunit in EC barrier function, ECIS experiments were performed in PP2A B α depleted and non-siRNA transfected HPAE and in HLMVE cells. We examined the effect of PP2A B α on thrombin induced changes of EC permeability. Cells were treated with either vehicle (0.1% DMSO), or thrombin (50 nM for HPAEC and 0.5 nM for HLMVEC) followed by monitoring TER for 3-10 hours (Figure 4A, B). Our results show that PP2A B α depletion decreases TER alone in HPAEC, but not in HLMVEC. Thrombin increases permeability of both cell types; furthermore the lack of PP2A B α significantly exacerbated the thrombin-induced decrease in TER and delayed the recovery in HLMVE cells or completely abolished TER recovery after thrombin in HPAE cells (Figure 7.4 A, B). We detected a significantly longer time period from the addition of thrombin until 50% recovery of PP2A B α silenced HLMVEC compared to non-siRNA transfected HLMVEC (0.42 hour vs 0.27 hour, respectively, $P < 0.05$; Figure 7.3Bb) These findings indicate the direct involvement of PP2A B α in barrier protection against thrombin-induced EC barrier compromise.

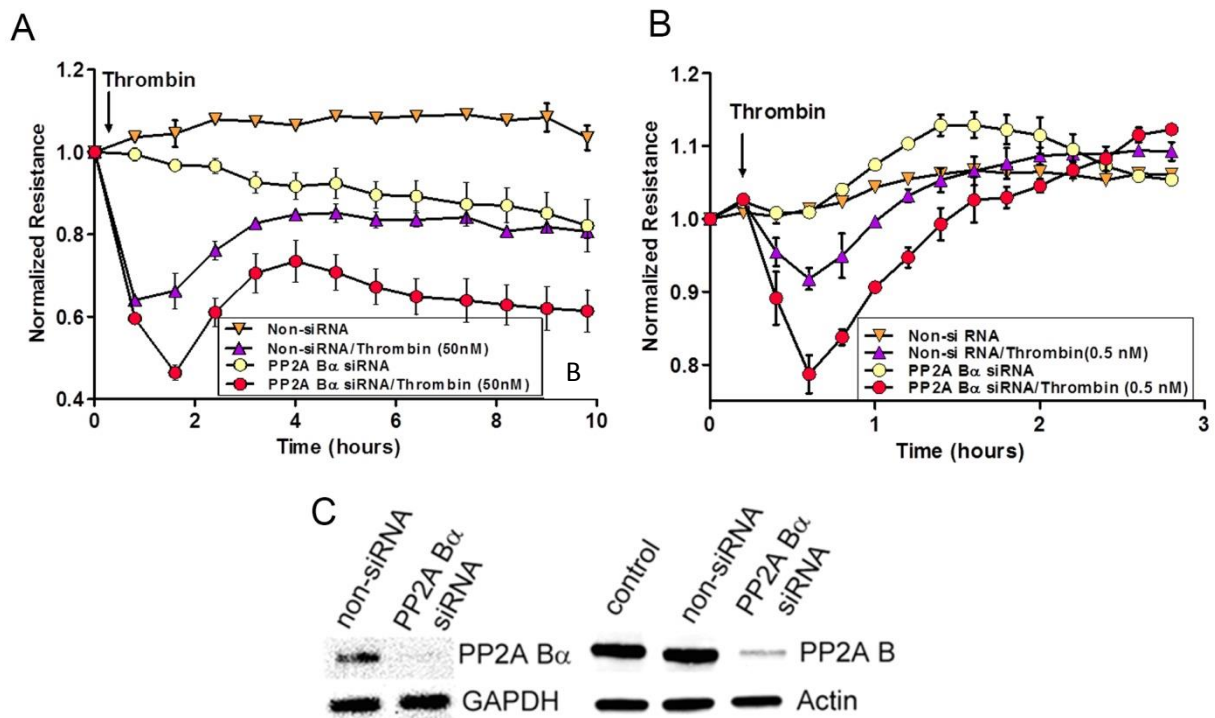


Figure 7.4. PP2A B α depletion affects endothelial cell barrier function of HPAEC and HLMVEC. HPAEC (A) and HLMVEC (B) were plated on gold microelectrodes and at 70-80% confluence they were transected with specific siRNA for PP2A B α or with non-siRNA. 72 hrs later TER measurements were started. Cells were treated with either vehicle (0.1% DMSO) or thrombin (A: 50 nM, B: 0.5 nM). Arrows indicate the time point when agonist or vehicle was added to the medium. PP2A B α depletion was confirmed by RT-PCR and Western blot (C) as described in Materials and Methods.

7.1.5. Subcellular localization of PP2A B regulatory subunit in ECs

To further investigate the role of the regulatory B α subunit of PP2A, we performed immunofluorescent staining to reveal its endogenous localization (Figure 7.5). Bovine pulmonary artery EC monolayer was double stained to visualize PP2A B (Figure 7.5A) and F-actin (Figure 7.5B). 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 as shown on the merged image (Figure 7.5C). 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.

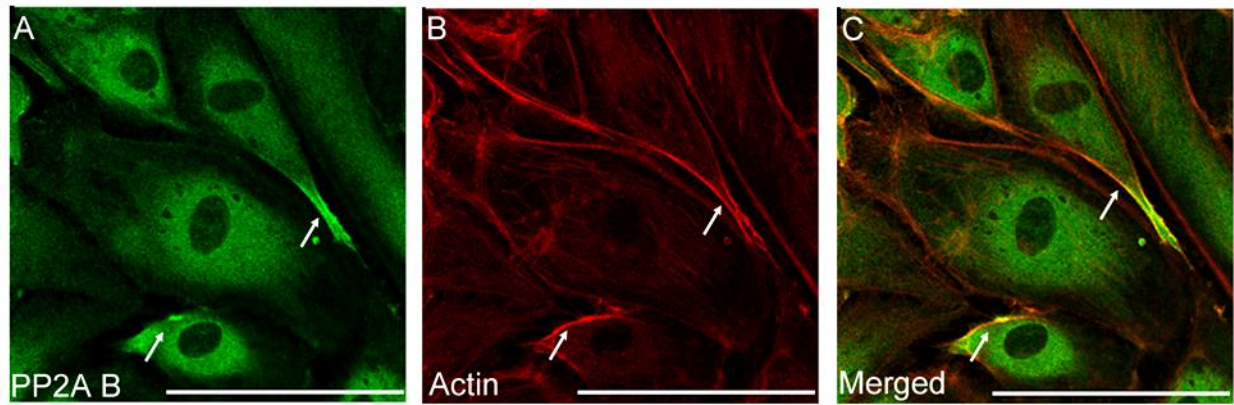


Figure 7.5. Regulatory B subunit of PP2A co-localizes with the cortical actin ring. BPAECs were plated on gelatin coated glass coverslips. After reaching confluency the monolayers were double stained to visualize PP2A B and actin microfilaments. Specific PP2A B antibody was used to stain the regulatory subunit (A) and Texas Red –phalloidin to stain actin (B). Arrows indicate the membrane localization of B subunit. Pictures were taken with an Olympus Fluoview FV1000 confocal microscope, scale bars: 200 μ m. A and B are parallel images of double-stained cells, panel C is merged image of A and B.

7.2. Regulation of endothelial junction assembly

7.2.1. PP2A B α regulatory subunit directly associates with adherent junction proteins

The partial membrane-like localization of the regulatory B subunit of PP2A (Figure 7.5) 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, β -catenin, strengthens the VE-cadherin- β -catenin interaction (Valenta et al., 2012). However the role of Ser/Thr phosphatases remained unclear. To explore whether PP2A is directly involved in AJs regulation we performed a pull-down experiment to search for possible binding partners of PP2A among AJ proteins. ECs are known as a difficult-to-transfect cell type with quite low transfection efficiency. Therefore, the PP2A B α /pcDNA3.1/V5-His construct was generated as described in Materials and Methods and appropriate controls were transfected into HEK cells using JetPEI transfection reagent (1:3 DNA:PEI ratio) (Figure 7.6). Over-expressed B α and control proteins were immobilized on anti-V5-agarose resin and after extensive washes cleared BPAEC lysates were loaded and the resin was further incubated. Bound proteins were eluted and analyzed with Western blot. We were able to identify adherent junction proteins along with the eluted B α using specific antibodies against β -catenin, VE-cadherin, and phosphorylated β -

catenin (Ser552) (Figure 7.7) providing evidence for the association of B α and EC adherent junction proteins. Importantly, neither the known PP2A binding protein, HSP27, nor the PP1 binding heat-stable phosphatase inhibitor 2 could bind the studied AJ proteins (Figure 7.7) in parallel, control pull-down assays confirming the specificity of the detected direct or non-direct binding of AJ proteins to B α .

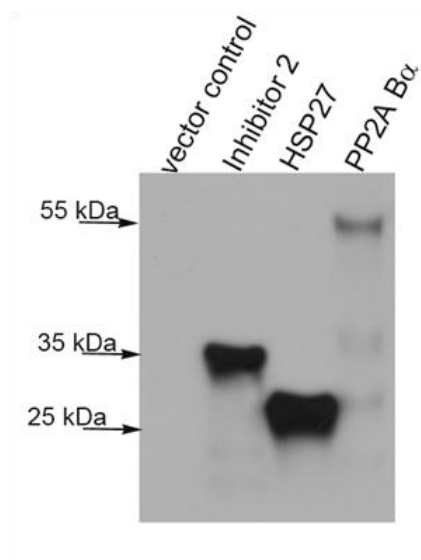


Figure 7.6. Overexpression of PP2A B α in HEK cells. HEK cells were transfected with PP2A B α pcDNA3.1/V5/His, inhibitor 2 pcDNA3.1/V5/His or HSP27 pcDNA3.1/V5/His mammalian expression constructs. Empty pcDNA3.1/V5His were also utilized as a negative control. Lysates of the transfected cells were analyzed with Western blot using anti-V5 antibody.

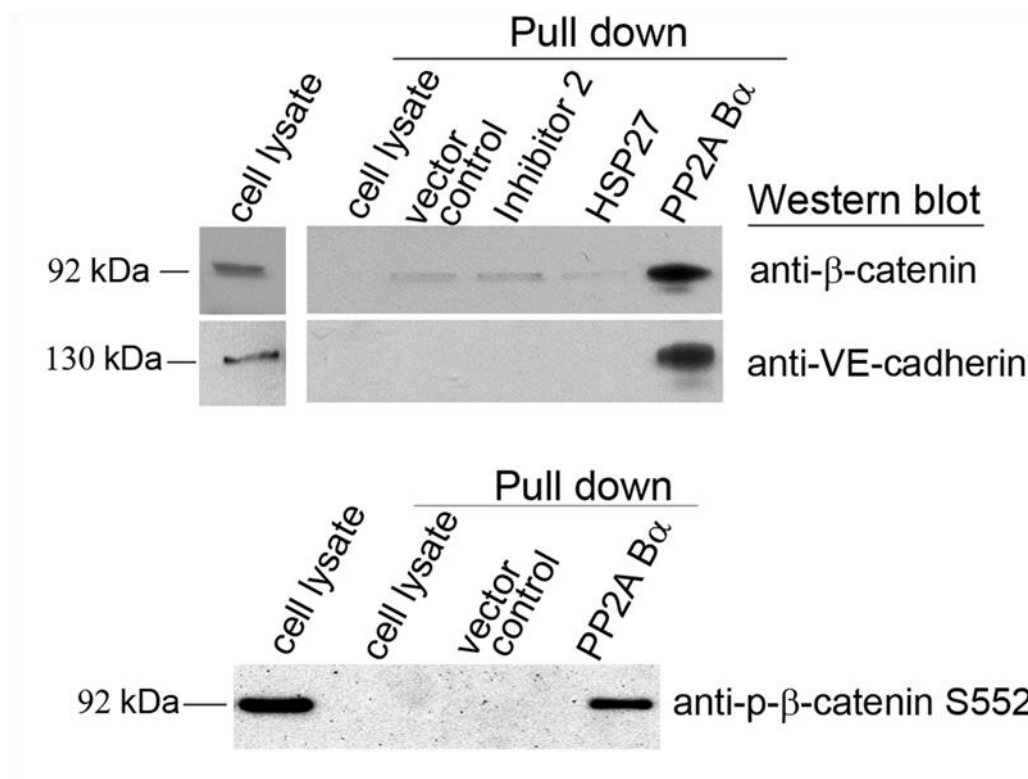


Figure 7.7. PP2A B α directly interacts with adherent junction proteins. Pull-down experiment was performed to examine the interaction between PP2A B α and AJ proteins. HEK cells were transfected either with pcDNA3.1/V5-His vector control or with mammalian expression constructs in the same vector. HEK cells were loaded onto anti-V5 agarose in order to immobilize V5 or V5-tagged inhibitor 2, HSP27, and PP2A B α on the resin. Unbound proteins were washed with PBS, and then the resin was incubated with BPAEC cell lysates. In addition, BPAEC lysate was incubated with anti-V5 agarose without any immobilized V5-tagged protein, as a negative control of the pull-down. After extensive washing, protein complexes were eluted by boiling the resin in 2x SDS sample buffer and analyzed by Western blot using β -catenin, VE-cadherin and phospho- β -catenin Ser552 specific antibodies. Untreated BPAEC cell lysate was also loaded in the first lane as a positive control.

7.2.2. Specific inhibition of PP2A increases the phosphorylation level of β -catenin and affects AJ assembly

Our previous experiment showed specific binding between PP2A and AJ proteins (VE-cadherin, total and phosphorylated β -catenin). To learn more about the role of PP2A in the regulation of cell junction assembly/disassembly, we employed specific PP2A inhibitors, okadaic acid (OA) or fostriecin, on HLMVE (Figure 7.8A) and OA on BPAE cells (Figure 7.8B). Fostriecin was used at 100 nM concentration for 1 hour and OA was used in 5 nM concentration for 90 min. After incubation with the chemicals cells were lysed and used for Western blot analysis. To examine the effect of the PP2A inhibitors on β -catenin phosphorylation we probed the membrane for specific antibody against phospho- β -catenin Ser552 as this phosphorylation site of β -catenin has been reported to be important in AJs assembly (Fang et al., 2007; Zhang et al., 2009). Fostriecin and okadaic acid treated cells show an increase in the phosphorylation level of Ser552 side chain in β -catenin (Figure 7.8A, B) suggesting that PP2A may have a role in the regulation of β -catenin dephosphorylation.

Total β -catenin was also visualized to check whether inhibition of PP2A causes any degradation of β -catenin. According to our results, fostriecin or OA treatment does not affect the protein level of β -catenin (Figure 7.8 A, B). Actin staining was also used as a loading control. Phosphorylation level of β -catenin was normalized to total β -catenin and quantified by densitometry of Western blots ($n=3$, $p < 0.05$ vs control). In addition, OA and fostriecin treated HLMVEC (Figure 7.8D) and OA treated BPAEC monolayers (Figure 7.8C) were immunostained for adherent junction proteins VE-cadherin (Figure 7.8Da,b,c and Ca,b) and β -catenin (Figure 7.8Dd,e,f and Cc,d). Both PP2A inhibitors induced interruption of continuous VE-cadherin staining at the cell periphery suggesting disruption of AJs (Figure 7.8Db,c and Cb). At the same time β -catenin staining at the edge of the cells was less pronounced after OA treatment (Figure 7.8Cc,d and Dd,e,f) without evident decrease of total protein amount (Figure 7.8A,B) suggesting some cellular redistribution of β -catenin. The effect of specific PP2A inhibition on the subcellular distribution of phospho- β -catenin was also examined. Immunofluorescent staining of EC using specific antibody revealed that β -catenin phosphorylated on Ser552 translocated from the cell membrane to the cytoplasm (Figure 7.8Dh,I and Cf).

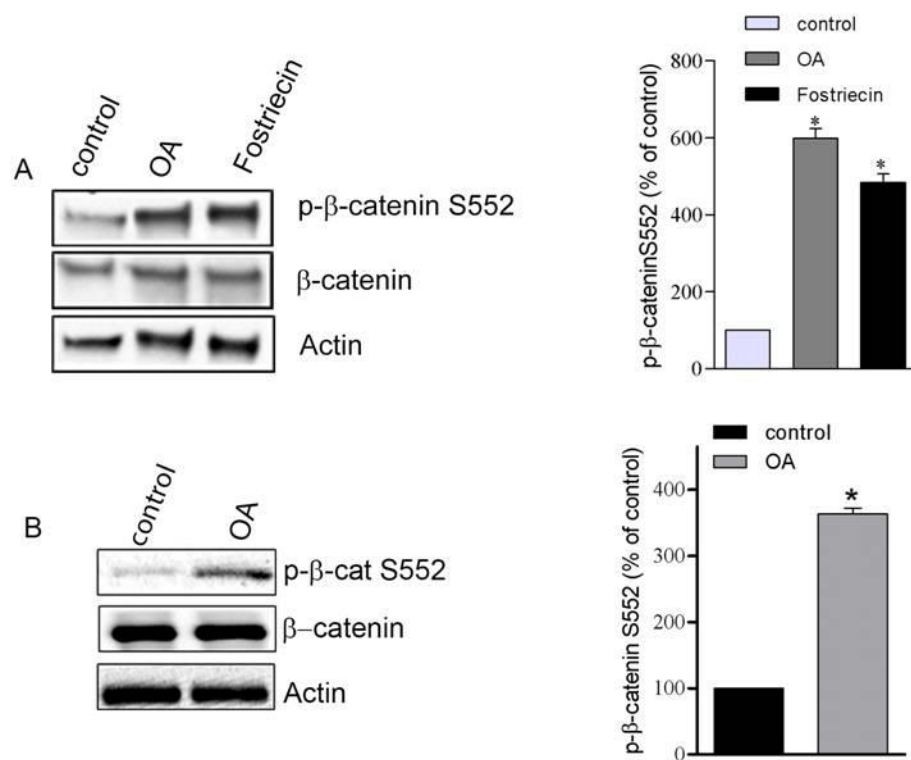
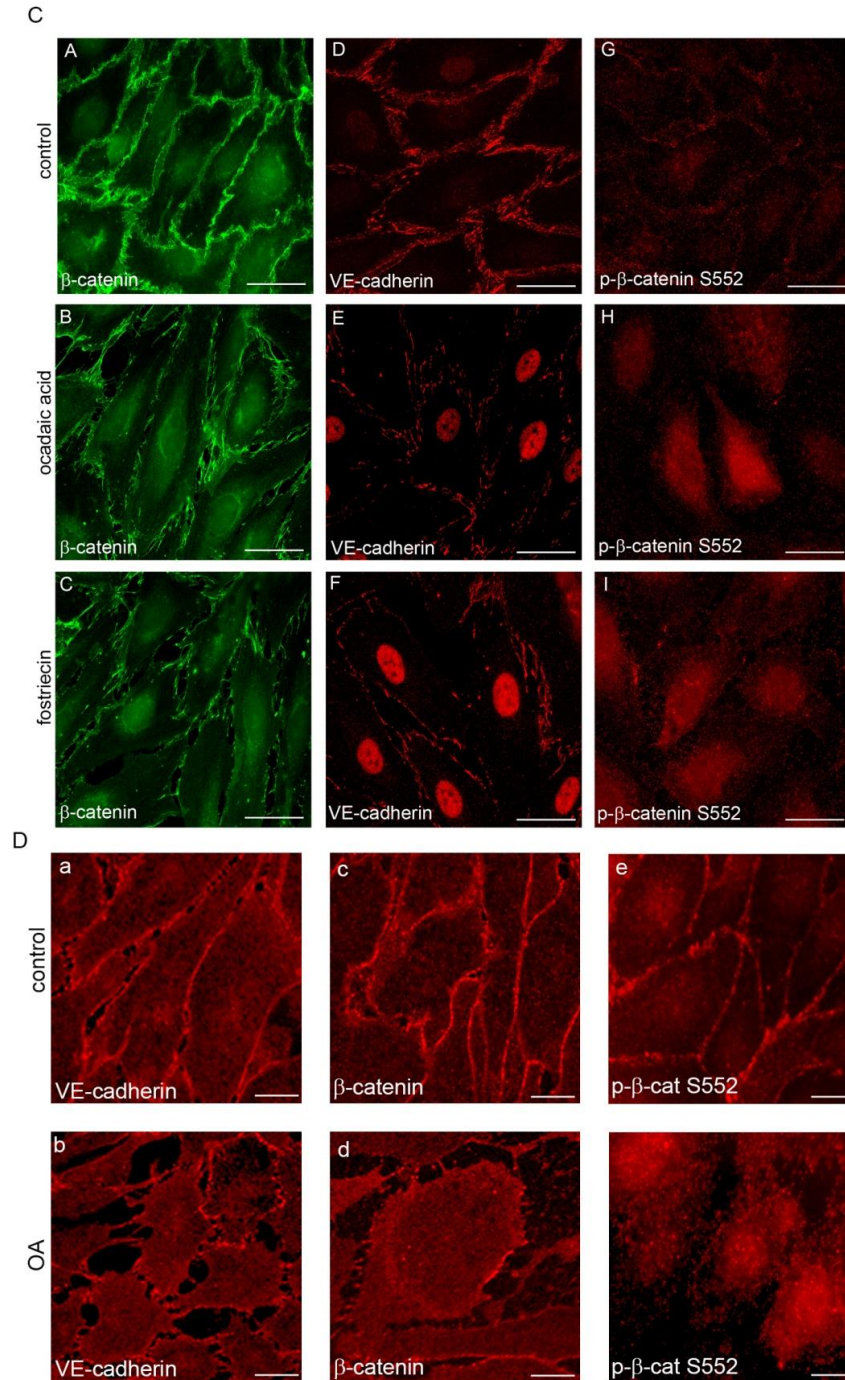


Figure 7.8. Fostriecin and okadaic acid treatments increase β -catenin phosphorylation and the disruption of AJ. BPAEC (B,C) and HLMVEC (A,D) monolayers were treated with 0.1% DMSO (control) or with 5 nM okadaic acid (OA) for 90 min. After that the cells were lysed for Western blot analysis (A, B) or fixed for immunofluorescent staining (C, D). (A, B) Phosphorylation level of β -catenin on Ser552 was analyzed by Western blot using specific antibody against phospho- β -catenin Ser552. β -catenin and actin were also detected as loading controls. Phosphorylation level of β -catenin was normalized to total β -catenin and quantified by densitometry ($n=3$, $p<0.05$).



(C,D) HLMVE and BPAE cells were treated with vehicle (Ca,d,g and Da,c,e) 5 nM okadaic acid (OA) for 90 min (Cb,e,h and Db,d,f) or with 100 nM fostriecin for 1 hour (Cc,f,i). After that the cells were fixed for immunofluorescent staining. Antibodies against VE-cadherin (Ca-c and Da-b), β -catenin (Cd-f and Dc-d) and phospho- β -catenin Ser552 (Cg-i and De-f), were used to detect the subcellular distribution of adherent junction proteins. The nuclear staining of the VE-cadherin is unspecific background staining. Pictures were taken with a Zeiss Axiolab microscope, scale bars: 200 μ m.

7.2.3. PP2A B α regulates β -catenin dephosphorylation in ECs

Our results indicated that PP2A inhibition negatively affected the adherens junction assembly and the lack of PP2A activity enhanced the phosphorylation of β -catenin (Figure 7.8). Furthermore we showed direct interaction between PP2A B α and AJs proteins. Next we examined the effect of specific depletion of PP2A B α on AJs. We hypothesized that the regulatory subunit targets PP2A to the cell periphery where it is able to interact with β -catenin. PP2A B α depleted cells and non-siRNA transfected cells were lysed and lysates were used for Western blot. Similarly to the effects of okadaic acid and fostriecin, the lack of B α regulatory subunit significantly elevated the level of phospho- β -catenin Ser552 (Figure 7.9A). In addition, we observed an inverse correlation between the protein levels of B α and the phosphorylation level of phospho-S552- β -catenin (Figure 7.9A). Membranes were probed for PP2A B to confirm the efficiency of the depletion. According to the statistical analysis of the Western blot, the depletion was about 70% (Figure 7.9A). In the next set of experiments, immunofluorescent staining was performed on PP2A B α depleted and non-siRNA transfected BPAEC. The monolayers were double stained for β -catenin and for phospho- β -catenin Ser552. Depletion of PP2A B α led to the translocation of β -catenin from the cell membranes to the cytoplasm and caused the disruption of AJs (Figure 7.9B). Interestingly, almost the entire amount of phospho Ser552- β -catenin in PP2A B α -depleted cells was located in the cytoplasm, but not at the cell edges. These data suggested that PP2A B α may be important for integrity of AJs, which is consistent with our data indicating that depletion of this subunit leads to EC barrier compromise (Figure 7.4). Overall, these data indicate the involvement of PP2A in the regulation of β -catenin phosphorylation level and cellular distribution, which affect AJ assembly and EC permeability.

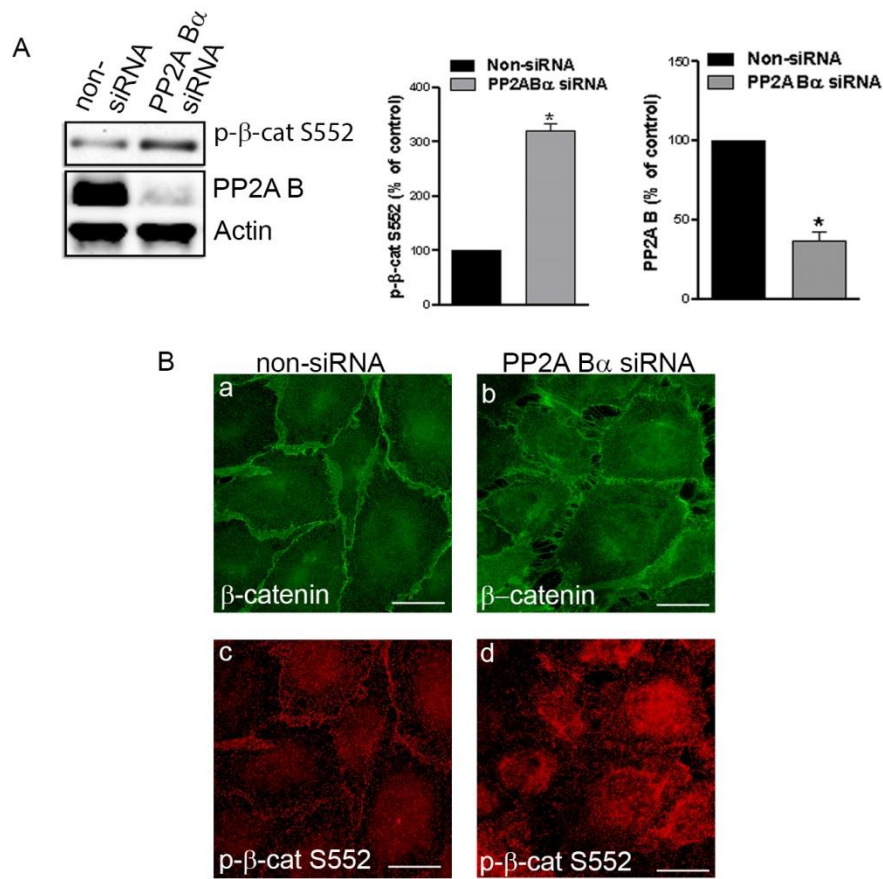


Figure 7.9. The role of PP2A B α in AJ regulation. BPAECs were transfected with specific PP2A B α siRNA and with non-silencing RNA. (A) Transfected lysates were used for Western blot analysis. Membranes were probed with phospho-β-catenin Ser552, PP2A B, and actin specific antibodies. Densitometric quantification of data are also demonstrated on panel A ($n = 3$, $p < 0.05$) (B). Images show immunofluorescent staining of PP2A B α specific siRNA and non-siRNA transfected cells with anti-β-catenin (a,b) and anti-phospho-β-catenin Ser552 (c,d) antibodies. Pictures were taken with a Zeiss Axiolab microscope, scale bars: 200 μm.

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

7.3.1. Investigation of putative binding partners for CPI-17

CPI-17 is known as a specific inhibitor of MP (Eto et al., 1995) 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 (Eto et al., 1995; Kitazawa et al., 2000; Pang et al., 2005). Data of literature suggested that PP2A can be potentially involved in MP activation via dephosphorylation/deactivation of CPI-17 (Ito et al., 2004), in consequence opposing EC contraction and permeability increase. Previously published data from Verin's group demonstrated the expression of endogenous CPI-17 in human lung microvascular endothelial cells and its involvement in the regulation of EC cytoskeleton and permeability (Kolosova et al., 2004). 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 (screening experiments were performed by Djanybek M. Adyshev) (Kim et al., 2013). Human cDNA whole lung 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 (Table 2.) are known, based on previous reports, to have function in actin cytoskeleton organization and cell adhesion (Aberle et al., 1994a; Van Troys et al., 1999).

Protein	GenBank accession #	Position of binding fragment, aa	Protein length, aa	Cellular process in which the protein is involved	Ref.
Plectin isoform 1	NP_000436	4457-4574	4574	Cytoskeleton-membrane attachment in the different tissues. Intermediate filament-associated protein that interlinks all three major protein cytoskeletal systems.	(Aberle et al., 1994a)
Alpha spectrin	NP_003118	2163-2472	2472	Cytoskeletal membrane protein. Links membrane proteins to the actin and microtubule filamentous skeleton.	(Bennett and Baines, 2001 ; Hartwig, 1994)
OK/SW-CL.16	BAB93516	1-134 35-134	134	Might act as a linker between -actinin-4 and some SH3 domain-containing signaling proteins.	(Togashi et al., 2007a)
Gelsolin isoform a	NP_000168	55-462	782	Control actin assembly, severing and capping of actin filaments. Plays role in cell motility and control of apoptosis.	(Silacci et al., 2004)
Junction plakoglobin	AAH00441	458-745	745	Plays a role in two intercellular junctions, the adherens junction and the desmosomes.	(Knudsen and Wheelock, 1992)

Table 2. Putative CPI-17 binding partner proteins

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 as described in the Materials and Methods. 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 (OK/SW-CL.16 was excluded because there is no commercially available antibody against this protein) 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 to the cytoskeletal regulation. We could confirm the interaction between overexpressed CPI-17 and plakoglobin by IP in lung endothelium (Figure 7.10A). Plakoglobin (γ -catenin) is an adherens junction protein, highly homologous to β -catenin. Plakoglobin plays an important role in cadherin/catenin complex assembly, as a linker between this complex and F-actin cytoskeleton (Aberle et al., 1994b). PKC activator PMA evokes the phosphorylation of

CPI-17 on Thr38 (Kolosova et al., 2004). We also examined the effect of PMA on CPI-17 plakoglobin interaction. As shown in Figure 7.10B, the binding affinity between CPI-17 and plakoglobin was decreased in the cells that were pretreated with PMA (0.1 μ M, 30 minutes). 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 β -catenin, VE-cadherin and p120 catenin, however, only binding of plakoglobin was detected, further demonstrating the specificity of CPI-17-plakoglobin interaction (Figure 7.10C). 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 (Fig 7.10C) as it was expected.

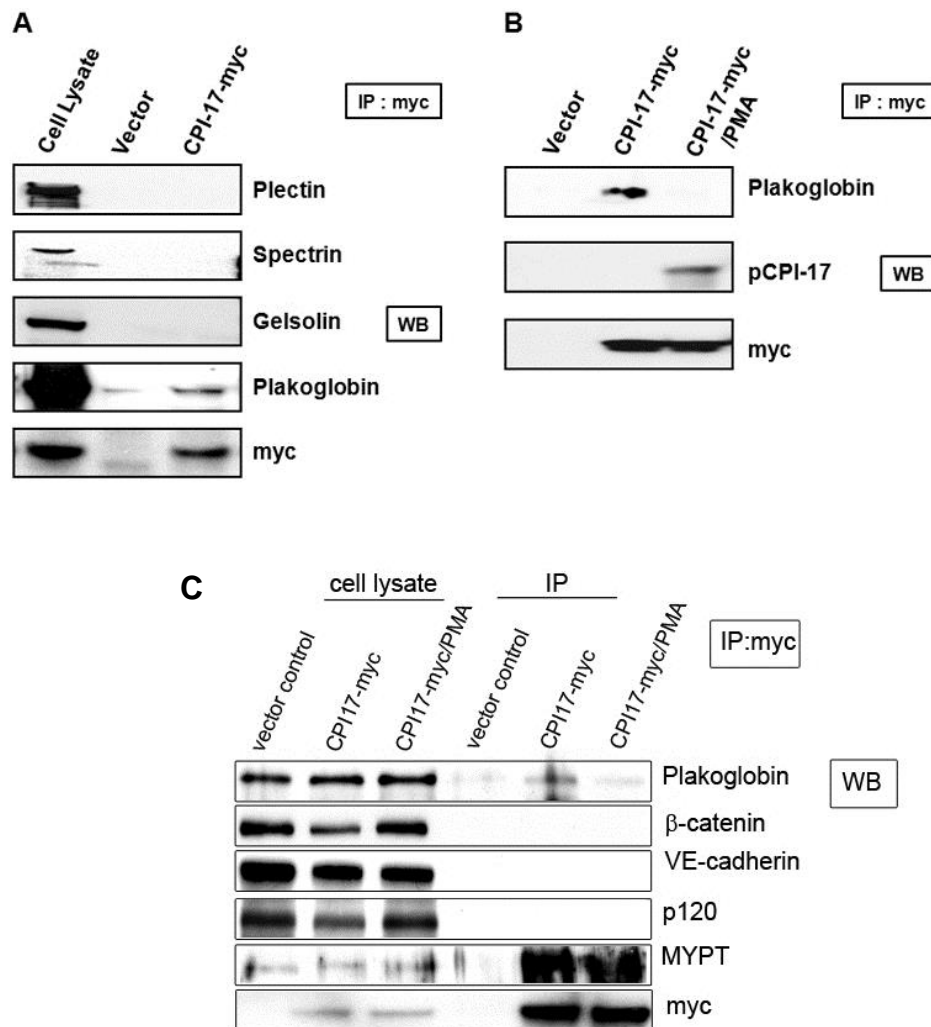


Figure 7.10. Putative CPI-17 interacting partners in microvascular endothelial cells. (A) Mammalian expression vector construct of CPI-17 with c-myc tag was transiently transfected into HLMVEC. 72 hours later lysates of the harvested cells were immunoprecipitated (IP) with anti-myc antibody, and then were subjected to Western blot analysis with antibodies against plectin, spectrin, gelsolin, plakoglobin, and c-myc. CPI-17-transfected cell lysates were used for Western blotting as a control of IP efficiency. (B) The effect of PMA-induced phosphorylation of CPI-17 on the binding affinity of plakoglobin was also examined by using phospho-CPI-17 antibody. Cells were pretreated with 0.1 μ M PMA for 30 minutes. After washing with PBS, IP and following Western blotting were conducted using anti-plakoglobin, -phospho-CPI-17, and -myc antibodies. (C) To confirm the CPI-17-plakoglobin interaction specificity, CPI-17-transfected cell lysates (control and PMA treated) and empty c-myc vector-transfected cell lysates were immunoprecipitated with anti-c-myc Affinity gel. Western blot membranes were probed with antibodies against plakoglobin, β -catenin, VE-cadherin, p120 catenin, MYPT1, and c-myc. Aliquots of cell lysates of vector control, CPI-17- myc, and CPI-17-myc/PMA were also loaded as controls.

7.3.2. 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 (0.1% DMSO) or with PMA (0.1 μ M 30 min) and stained for plakoglobin or c-myc. We were able to detect co-localization of recombinant CPI-17 and endogenous plakoglobin (Figure 7.11A-C) at the attachment sites of neighboring cells. Although, increased plakoglobin staining was observed in PMA treated cells, the CPI-17 staining became rather scarce in the cell membrane (Figure 7.11D-F).

These stainings provided further evidence 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.

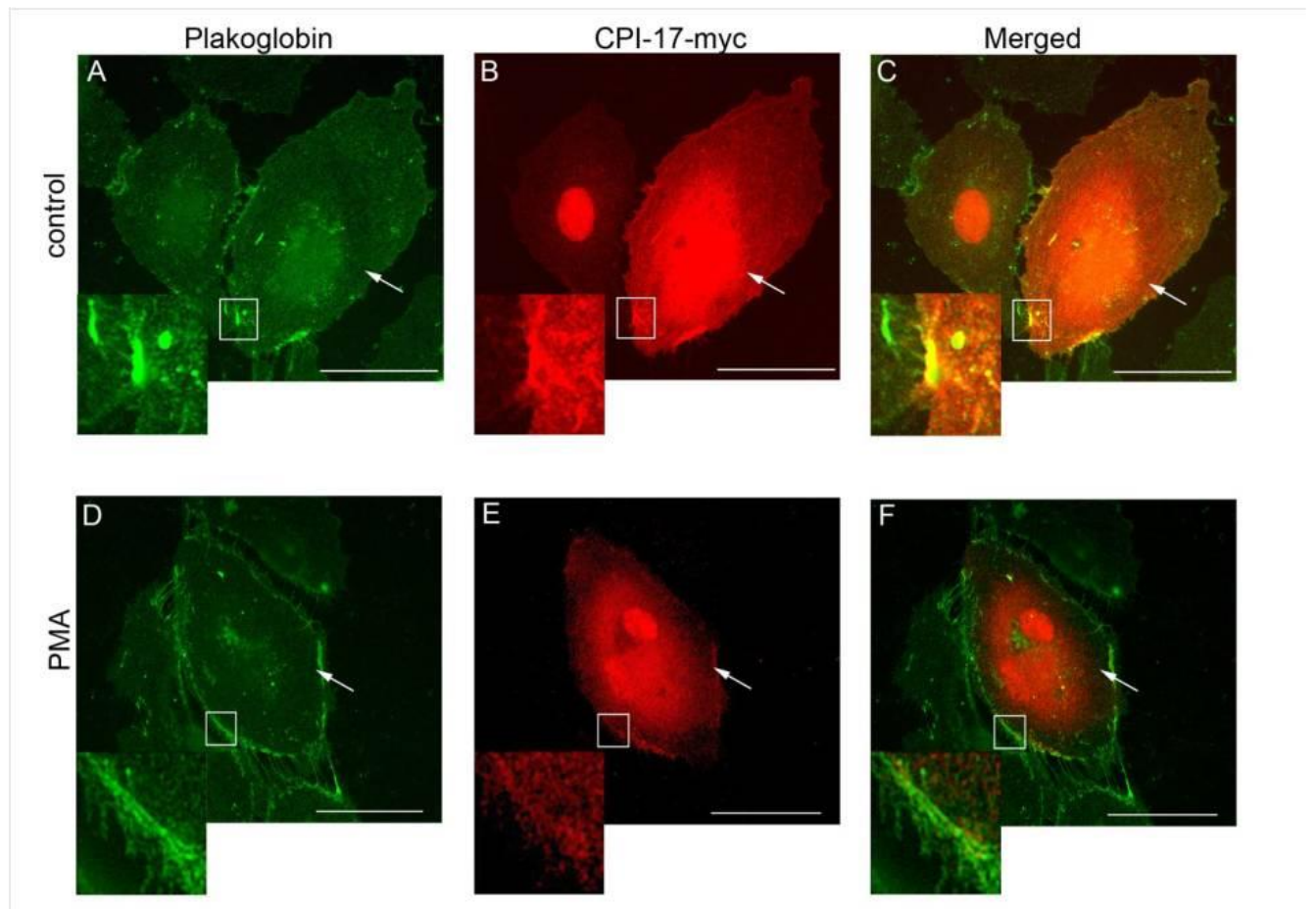


Figure 7.11. Immunofluorescent staining revealed co-localization of recombinant CPI-17 and plakoglobin. HLMVEC were double stained with c-myc antibody to visualize CPI-17 (B, E) and specific antibody against plakoglobin (A, D). Double stained images are shown in parallel. Transfected cells are indicated with arrows. Panel C is a merged image of panels A and B, and panel F is a merged image of D and E. Co-localization of CPI-17 and plakoglobin on panel C is shown enlarged. Similar area (lack of co-localization) is shown enlarged (panel F). Scale bars: 200 μm . Same experiments were repeated three times.

8. DISCUSSION

Lung endothelium regulates movement of fluid and macromolecules between the blood and the interstitial space. The integrity of pulmonary endothelial cell monolayer, which is heavily dependent upon the EC cytoskeleton network (actin filaments, microtubules and proteins of cell junctions) (Bazzoni and Dejana, 2004; Dudek and Garcia, 2001), is a critical requirement for tissue and organ homeostasis. Disruption of endothelial barrier results in the movement of solutes and macromolecules into the interstitium and pulmonary air spaces causing pulmonary edema (Pugin, 1999). It is a common feature of potentially lethal pathophysiological conditions such as ALI and ARDS (Ware and Matthay, 2000). Despite the importance of endothelial barrier limited information exists concerning mechanisms involved in EC barrier function preservation.

PP2A is a major Ser/Thr phosphatase responsible for dephosphorylation and regulation of many molecular targets (Janssens and Goris, 2001). A growing body of evidence indicates association between PP2A and cytoskeletal structures, such as microtubules (MT). The essential role of PP2A in MT stability was shown by several authors (Hiraga and Tamura, 2000; Sontag et al., 1995; Sontag et al., 1999). Data from our lab clearly demonstrated tight association of PP2A with microtubules in endothelium (Tar et al., 2004). Regardless of the large amount of information concerning the link between PP2A activity and the cytoskeleton, the exact role of PP2A in the regulation of EC cytoskeleton and EC permeability has remained largely unexplored.

To further clarify the effect of PP2A on endothelial cell cytoskeleton organization different cell types were used, mostly due to technical limitations. Two types of macrovascular cell lines, bovine pulmonary artery endothelial cells (BPAEC) and human pulmonary artery endothelial cells (HPAEC), and a microvascular cell line, human lung microvascular endothelial cells (HLMVEC). The macro- and the microvascular cells share many similarities in their characteristics and in physiological properties. The majority of the macromolecule or fluid transport occurs through the capillaries, while inflammatory processes take place in the large vessels involving the macrovascular cells (Feletou, 2011). Overall, our results on the micro- and macrovascular cells do not show significant differences. Still, the minor alterations can be due to the different cell types.

Okadaic acid and fostriecin was applied on microvascular- and macrovascular endothelial cells to inhibit PP2A activity. Okadaic acid (OA) is a cytotoxic polyether derivative

of 38-carbon fatty acid isolated from *Halichondria okadai* and *H. mealnodoncia* sponges (Haystead et al., 1989). OA is a powerful inhibitor of type 1 and 2A protein phosphatases (Bialojan and Takai, 1988). It inhibits PP2A more efficiently ($IC_{50} = 0.1$ nM) compared to PP1 ($IC_{50} = 15-20$ nM) (Cohen et al., 1990). Fostriecin, an antitumor antibiotic produced by *Streptomyces pulveraceus*, is a potent inhibitor of PP2A (PP2A; IC_{50} 3.2 nM) and a weak inhibitor of type 1 (PP1; IC_{50} 131 μ M) Ser/Thr protein phosphatase (Walsh et al., 1997). Previously, our group used successfully OA (at 2-5 nM concentration) which substantially strengthened the effect of suboptimal concentrations of the MT disruptor, nocodazole on transendothelial electrical resistance (TER) and facilitated nocodazole-induced paracellular gap formation (Tar et al., 2004). Other studies also underline the effect of PP2A on cytoskeleton reorganization. For example Sontag and colleagues found that PP2A is associated with the interphase microtubules and the mitotic spindle in fibroblasts, epithelial cells, and neurons. Furthermore, OA induced microtubule dissociation and hyperphosphorylation of MT-associated protein tau (Sontag et al., 1995; Sontag et al., 1996). In addition, selective inhibition of PP2A with fostriecin provoked aberrant mitotic centrosome replication and spindle formation in CHO (chinese hamster ovary) epithelial cells (Cheng et al., 1998). Also, OA induced disorganization of actin filaments in NIH/3T3 fibroblast cells (Usui et al., 1999). In our experiments OA and fostriecin treatment caused alteration of endothelial cytoskeleton organization. The increased amount of actin stress filaments and the partial microtubule dissociation both suggest the critical role of PP2A in the regulation of EC cytoskeleton remodeling. In turn, previous data of our group demonstrated that 5 nM OA had no significant effect on HPAEC permeability (Verin et al., 1995). Moreover, immunofluorescence staining of F-actin did not show any detectable change provoked by OA (Tar et al., 2004). This controversy may be due to the differences in cell culture conditions. In both of the previously published works the human and bovine ECs were maintained in M199 containing 20% bovine serum and endothelial cell growth supplement. In contrast, in the present study we used MEM, according the manufacturer's recommendation, supplemented with only 10% of bovine serum to culture BPAEC for better comparability with serum-starved silencing conditions. This serum concentration was used by several other groups (Drew et al., 2010; Ludwig et al., 2005).

Previously our group examined the role of catalytic C and scaffolding A subunit of PP2A in EC barrier regulation. Human pulmonary artery endothelial cells were transfected with mammalian expression constructs of the A or C subunits. The overexpression of PP2A C or A and C together attenuated thrombin or nocodazole induced EC permeability decrease in TER,

indicating barrier protection (Tar et al., 2006). As the AB α C holoenzyme is the most abundant form of PP2A and B α is reported to be involved in several cellular functions we intended to further examine the role of this regulatory subunit on EC barrier function. Inhibition of PP2A activity by OA negatively affected the normal endothelial function; therefore we examined the effect of B α depletion on endothelial cells, to see whether the lack of this regulatory subunit will alter the cytoskeleton structure via modification of PP2A activity. The achieved reduction (~ 80%) of B α protein level by specific siRNA affected the organization of actin microfilaments by inducing stress fiber formation. More importantly, thrombin induced stress fiber formation became more dramatic in B α silenced cells compared to the non-silencing RNA treated controls suggesting that B α plays an important role in the barrier protective function of PP2A. Besides we studied the effect of specific B α silencing on EC permeability in control- and thrombin-challenged cells. Our results indicate that the depletion of this subunit substantially decreased basal TER and exacerbated thrombin induced EC permeability. These major findings were observed both in macrovascular HPAE and microvascular HLMVE cells. In the case of macrovascular cells PP2A B α depletion decreased TER recovery after the thrombin treatment. Less dramatic effect of depletion was observed in microvascular EC. The time period was significantly longer starting from thrombin treatment until 50% recovery of PP2A B α silenced microvascular endothelial cells compared to non-siRNA transfected cells. Collectively these data indicate that PP2A B α regulatory subunit of PP2A is critical for endothelial cells barrier maintenance.

The subcellular distribution of B subunit has been studied in numerous different tissue and cell types, but its endogenous localization in the endothelium has not been previously shown (Janssens et al., 2008; Mayer et al., 1991). We performed immunofluorescent staining of the endogenous endothelial B α , which revealed mainly cytoplasmic localization of this subunit in agreement with the previous findings in other cell types. In addition, we could observe co-localization of cortical F-actin and PP2A B at EC periphery suggesting that PP2A may play a role in the cortical actin and cell junction assembly.

Adherens cell junctions are essential in the maintenance of endothelial integrity providing connection between neighboring ECs, thus regulating endothelial barrier function. AJs' main components are the transmembrane VE-cadherin and its cytosolic binding partner, β -catenin. It is important to mention that the formation of the cadherin-catenin complex is largely dependent on phosphorylation and dephosphorylation events. The kinases involved in

the regulation of AJ proteins are studied in detail; however the role of phosphatases is less known. For example, the cytoplasmic domain of VE-cadherin is phosphorylated at Ser684,-686,-692 creating more interaction sites for β -catenin binding (Huber and Weis, 2001). The regulation of β -catenin is also highly dependent on phosphorylation. It is well established that phosphorylation of β -catenin by casein kinase I (CKI) on Ser45, and GSK3 β on Ser33/37, Thr41 leads to its ubiquitination and proteosomal degradation (Aberle et al., 1997; Rubinfeld et al., 1996). Wnt and other growth stimuli lead to the inactivation of GSK3 β , β -catenin will be less phosphorylated and translocates into the nucleus and binds to transcription factors, T-cell factor (TCF) and lymphoid enhancing factor-1 (LEF-1) involved in angiogenesis. In some cases phosphorylation of VE-cadherin or β -catenin has a negative effect on AJ complex assembly. For instance tyrosine phosphorylation on Y860 of VE-cadherin and Y654 on β -catenin leads to disassembly of the catenin-cadherin complex (Lilien and Balsamo, 2005).

Data of literature demonstrated the association the A and C subunits of PP2A with the E-cadherin- β -catenin complex in epithelial cells by immunofluorescent staining and Western blot (Usui et al., 1988). Disruption of cell contacts and induction of cell rounding mediated by okadaic acid, a specific inhibitor of PP2A suggests the functional role of PP2A in the maintenance of cell junction complexes (Takahashi et al., 2006). Furthermore, Su and colleagues demonstrated that PP2A dephosphorylates β -catenin in bovine cardiac muscle (Su et al., 2008). More recent data also demonstrated that B α regulatory subunit of PP2A controls β -catenin dephosphorylation during Wnt-signaling in *Drosophila* (Zhang et al., 2009).

According to our above mentioned findings combined with the literature data about the distribution of PP2A B in ECs, we speculate that this regulatory subunit may play a role in cell junction assembly. Therefore we investigated the possible interaction between AJ proteins and PP2A B. We utilized overexpressed B α and control recombinant proteins as baits in pull down experiments. VE-cadherin, β -catenin and phospho- β -catenin S552 were detected as binding partners for B α .

To further elucidate the connection between AJ proteins and PP2A/ PP2A B α subunit, we performed immunofluorescent staining. As it was expected, VE-cadherin and β -catenin appeared at the cell-cell contacts. Inhibition of PP2A by okadaic acid or fostriecin dramatically changed the localization of these proteins. We could observe the dissociation of adherens junctions and the relocation of β -catenin and VE-cadherin from the cell membrane to the

cytoplasm and nuclei. Similarly, depletion of PP2A B α resulted in loss of cell contacts and the redistribution of AJ proteins. Our findings are supported by previously published data (Serres et al., 1997). They reported the hyper-phosphorylation of β -catenin on Ser/Thr residues caused by okadaic acid and calyculin A treatment followed by the loss of cell contacts in human keratinocytes (HaCaT). They also presented data on E-cadherin phosphorylation mediated by Ser/Thr kinase, casein kinase 2 (CK2) which led to the disruption of adherens junctions in HaCaT (Serres et al., 2000; Serres et al., 1997). In addition, thrombin induced release of β -catenin and p120 catenin from the cell membrane has been described recently in human umbilical vein ECs (Beckers et al., 2008).

In our experiments, immunofluorescent staining showed alteration in the distribution of phospho- β -catenin caused by the inhibition of PP2A activity with OA or fostriecin, or the depletion of PP2A B α . Treatments with the inhibitors or B α silencing increased the phosphorylation level of β -catenin at Ser552 and caused its translocation from the membrane to the cytoplasm and the nucleus. Western blot analysis further confirmed the results of immunofluorescence staining. These results suggest that B α targets PP2A to the cell membrane where it could control the phosphorylation of β -catenin. The Ser522 phosphorylation of β -catenin is not implicated in the Wnt signaling. In quiescent cells the phosphorylation level of this Ser residue was very low and phospho- β -catenin Ser552 could be detected at the cell periphery of adjacent ECs. As recent papers reported AKT is responsible for the phosphorylation of β -catenin at Ser522, which leads to its dissociation from cell contacts (Fang et al., 2007; Taurin et al., 2008).

Several other publications highlight the importance of PP2A in the cadherin-catenin complex formation in mice embryogenesis and in epithelial cells in accordance with our findings (Gotz et al., 2000; Nita-Lazar et al., 2010; Takahashi et al., 2006). Despite of its stabilizing effect on AJs, PP2A has been shown to negatively regulate the tight junction assembly (Nunbhakdi-Craig et al., 2002). At this point it is important to mention that the adherens junction assembly precedes tight junction formation and in some *in vivo* cases cadherin is required for the formation of TJs, as it controls the recruitment of ZO-1 to TJ complexes (Tunggal et al., 2005).

Collectively our results demonstrate that PP2A B α plays a crucial role in EC cytoskeleton arrangement and endothelial barrier function. Also, it is involved in the regulation

of adherens junction assembly via regulation of dephosphorylation of β -catenin in endothelial cells.

Earlier published data and our new results demonstrated that myosin phosphatase (MP) and PP2A are directly involved in EC barrier regulation (Kolosova et al., 2005; Tar et al., 2004; Tar et al., 2006). Also, PP2A activation leads to the dephosphorylation which substantially decreases the inhibitory effect of CPI-17 on MP (Hersch et al., 2004; Ito et al., 2004). However, the precise physiological function of PP2A/CPI-17 signaling in MP regulation in endothelium is currently unexplored. PKC potentiated inhibitory protein, CPI-17 was first identified as a myosin phosphatase inhibitor in smooth muscle cells (Eto et al., 1997). The expression of endogenous CPI-17 in human LMVEC and its involvement in the regulation of EC cytoskeleton and permeability have already been explored (Kolosova et al., 2004). Our collaborators performed bacterial two hybrid screening to find putative binding partners for CPI-17. Human lung cDNA library was used in this screening, because human pulmonary EC cDNA library was not available. *E. coli* two hybrid system was developed 15 years ago, and since then successfully and extensively used by many research groups (Karimova et al., 1998). Its advantage is that bacteria grow faster and have higher transformation efficiency than yeast, make it possible to screen large number of interactions in a short time. The major disadvantage is that in bacteria the posttranslational modifications, such as phosphorylation, are missing.

Fourteen binding partners were identified which possibly interact with CPI-17. From the fourteen putative binding partners, five proteins were selected, plectin 1 isoform 1, alpha II spectrin, OK/SW-CL.16, gelsolin isoform a, and plakoglobin, based on their ability to regulate actin cytoskeleton and cell junctions (Aberle et al., 1994b; Bennett and Baines, 2001; Hartwig, 1994; Knudsen and Wheelock, 1992; Togashi et al., 2007). The remaining nine proteins were excluded because of their less relevance in cytoskeleton organization and cell junction regulation. To confirm interactions, co-immunoprecipitation experiments were performed in human lung microvascular endothelial cells. OK/SW-CL.16 was excluded from IP experiments due to the lack of commercially available antibody against this protein. We were able to confirm specific interaction between recombinant CPI-17 and plakoglobin in lung microvascular endothelial cells, but no interaction was found between CPI-17 and spectrin, plectin or gelsolin.

Plakoglobin is an intracellular binding partner for VE-cadherin in endothelial cells and its main function is to stabilize the AJ complex (Aberle et al., 1994b; Dejana, 2004).

Plakoglobin is highly homologous to β -catenin. Through α -catenin, plakoglobin is in connection with actin binding proteins, like α -actinin and ZO-1 (Weis and Nelson, 2006).

Interestingly MYPT was not revealed by the two hybrid screening as a target for CPI-17. This can be explained by the fact that the interaction between CPI-17 and its binding partner may be affected by the tertiary structure of each protein, or possibly by phosphorylation which was not available in bacteria. However, we were able to detect their interaction with co-immunoprecipitation, as it was expected. We investigated whether the binding affinity between CPI-17 and plakoglobin can be affected by phosphorylation using PMA as agonist. PMA is a known PKC activator, it causes phosphorylation of CPI-17, and it affects endothelial barrier dysfunction (Kolosova et al., 2004). We detected significantly less interaction between plakoglobin and phospho-CPI-17 due to PMA treatment. In addition, our immunofluorescence staining provided further evidence for the negative effect of phosphorylation on the interaction of CPI-17 and plakoglobin as co-localization of these two proteins was revealed in untreated but not in PMA-treated HLMVEC.

Our results in concert with literature data help to better understand the endothelial barrier regulation and cytoskeleton organization by protein phosphatases. Our findings suggest that CPI-17 may play a dual role in endothelial barrier regulation (Figure 8.1). In quiescent endothelial cells CPI-17 is dephosphorylated and inactive, associated with adherens junctions via plakoglobin. In these circumstances, PP2A has significant basal activity which keeps CPI-17 in dephosphorylated/inactive status. We speculate that under these conditions CPI-17 may contribute to the stabilization of adherens junctions. Barrier compromising agents like gram negative bacterial lipopolysaccharide (LPS), thrombin or histamine activate PKC causing CPI-17 phosphorylation and activation. Activated CPI-17 dissociates from AJs and induces the inhibition of myosin phosphatase leading to endothelial cell contraction followed by permeability increase.

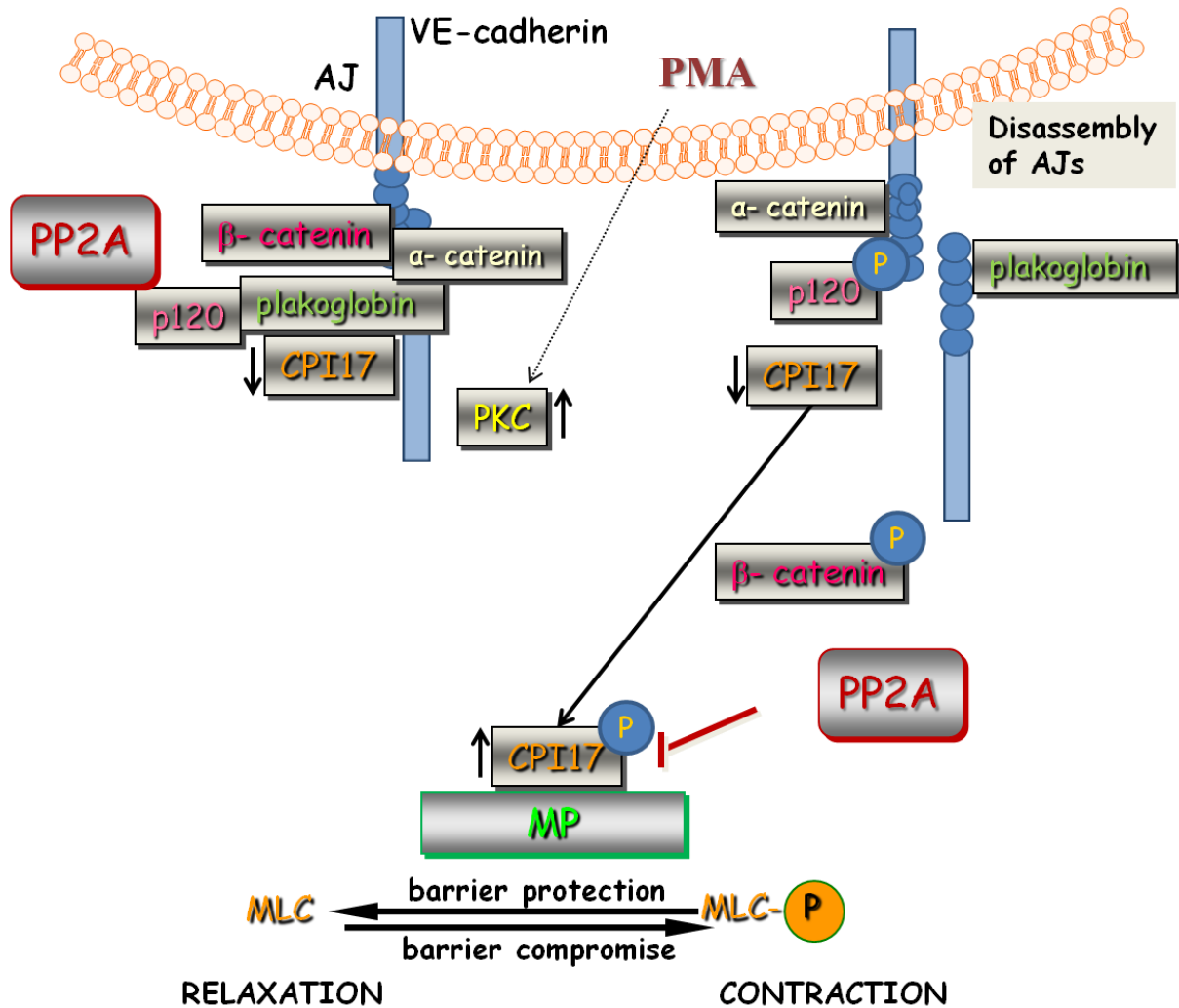


Figure 8.1. The role of PP2A in endothelial barrier protection. In quiescent cells CPI-17 is linked to adherens junctions through plakoglobin where CPI-17 is unphosphorylated and less active (\downarrow). Due to PMA stimuli PKC is activated (\uparrow) and is able to phosphorylate CPI-17 on Thr38 leading to its activation (\uparrow) and dissociation from the adherens junction complex. In the cytosol phospho-CPI-17^{Thr38} binds to myosin phosphatase and inhibits MP activity resulting in increased myosin light chain (MLC) phosphorylation, actin stress fiber formation and cell contraction. PP2A stabilizes AJ complex by dephosphorylating β -catenin, furthermore can reverse cell contraction, by dephosphorylating and inactivating CPI-17.

Taken together, this present work reinforces previous findings that PP2A has a positive regulatory role in endothelial barrier function. We demonstrated that PP2A associated with β -catenin via the regulatory PP2A B α subunit. More importantly, that specific depletion of this subunit exacerbates thrombin-induced endothelial permeability increase. Our results indicate that the regulation may occur through direct dephosphorylation of AJ target proteins of PP2A, and indirectly via PP2A-CPI-17-mediated regulation of myosin phosphatase and adherens junction arrangement (Figure 8.1). Collectively, these results expand our current understanding of the molecular mechanisms of Ser/Thr phosphatase-mediated EC barrier.

9. 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 α regulatory subunit. Inhibition of PP2A with OA and fostriecin affected actin arrangement and caused microtubule dissolution. Depletion of the B α subunit had a similar effect, as it led to actin reorganization. Moreover the lack of PP2A B α significantly exacerbated thrombin-induced increase in EC barrier permeability. AJ proteins VE cadherin and β -catenin were detected as binding partners of B α with pull down assay. Inhibition of PP2A caused the redistribution of β -catenin and VE-cadherin, from the cell junctions to the cytosol and nucleus and resulted in phosphorylation of β -catenin. Similarly, the depletion of B α affected the F-actin organization in EC, and lead to the disruption of AJs, parallel with β -catenin phosphorylation on Ser552. The effect of B α 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 permeability. Plakoglobin, an AJ protein stabilizing cell junctions, was revealed as a putative binding partner 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 α has a functional role in the barrier protective function of PP2A and in the regulation of AJ proteins via the dephosphorylation of β -catenin. Moreover, our data suggest that CPI-17 can interact with major elements of the cytoskeleton through its binding partner plakoglobin and have a possible regulatory role in EC barrier function.

10. ÖSSZEFOGLALÓ

A jól működő vaszkuláris endotél barrier funkció nagymértékben függ a citoszkeletális és adherens sejtkapcsoló (AJ) fehérjék Ser/Thr oldalláncainak foszforilációjától. Munkacsoportunk korábban kimutatta, hogy a protein foszfatáz 2A (PP2A) katalitikus és szerkezeti alegysége jelentős szerepet játszik a barrier funkció fenntartásában. Irodalmi adatok alapján tudjuk, hogy a miozin foszfatáz (MP) szabályozó szerepet tölt be a sejtkontrakcióban. Munkánk során célul tűztük ki a PP2A B α regulátor alegység illetve a PP2A egyik szubsztrátja, a specifikus MP inhibitor, CPI-17 szerepének tisztázását a barrier funkció szabályozásában.

Makro- és mikrovaszkuláris endotél sejteket PP2A gátlószerekkel (okadánsav (OA) és fosztriecin) kezelve az aktin filamentumok átrendeződését és a mikrotubulusok destabilizálóját figyeltük meg. Hasonlóképpen aktin stressz kábelek kialakulását tapasztaltuk a PP2A B α csendesítése során, ami trombin kezelés hatására fokozódott. Transzendotél ellenállás méréseink fokozott endotél permeabilitás növekedést mutattak a B α csendesített sejtekben trombin kezelés hatására a nonsi-RNS transzfektált sejtekhez képest. Pull-down kísérlettel sikerült specifikus kölcsönhatást kimutatni a B α és két adherens sejtkapcsoló fehérje, a VE-cadherin és β -catenin között. Az OA és fosztriecin kezelés a VE-cadherin és β -catenin transzlokációját okozta sejtmembránból a citoplazmába, továbbá a β -catenin hiperfoszforilációját tapasztaltuk. Immunfloreszcens és Western blot kísérleteink azt mutatták, hogy a specifikus PP2A B α depléción negatívan befolyásolta az aktin filamentumok elrendeződését, és az adherens sejtkapcsolatok széteséséhez vezetett, ezzel párhuzamosan a β -catenin S552 foszforilációját okozta.

A PP2A defoszforilálja és ezáltal a CPI-17 fehérje inaktivációját okozhatja, azonban a CPI-17 és a citoszkeleton kapcsolatáról jelenleg keveset tudunk. A CPI-17 fehérjét a PKC enzim foszforilálja és fokozza a CPI-17 gátló hatását a MP-al szemben. Korábbi vizsgálatok igazolták a CPI-17 expresszióját HLMVE sejtekben, és szerepét az endotél barrier funkcióban. A bakteriális két-hibrid szűrés során talált lehetséges kölcsönható partnerek közül ko-immunprecipitációval sikerült igazolnunk a plakoglobin és a CPI-17 kapcsolatát HLMVE sejtekben. PMA kezelés hatására azonban a két fehérje között csökkent kölcsönhatást tudtunk kimutatni.

Eredményeink alapján arra következtetünk, hogy a B α regulátor alegység jelentős szabályozó szereppel bír a citoszkeleton és az AJ fehérjék defoszforilációjának szabályozásában. Kimutattuk, a CPI-17 és a plakoglobin kölcsönhatását mikrovaszkuláris endotél sejtekben. Eredményeink azt igazolják, hogy a CPI-17 szerepe az AJ stabilizálásában és a barrier funkció szabályozásában foszforiláció függő folyamat, amelyben a PP2A fontos szerepet tölthet be.

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Register Number: DEENKÉTK/266/2013.

Item Number:

Subject:

Ph.D. List of Publications

Candidate: Anita Kása

Neptun ID: FJJQ0G

Doctoral School: Doctoral School of Molecular Medicine

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.
Microvasc. Res. Epub ahead of print (2013)
DOI: <http://dx.doi.org/10.1016/j.mvr.2013.05.003>
IF:2.929 (2012)
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>
IF:2.929 (2012)

List of other publications

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.
Am. J. Physiol.-Lung Cell. Mol. Physiol. Article in press, 2013.
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4. Czikora, I., Kim, K., **Kása, A.**, Bécsi, B., Verin, A., Gergely, P., Erdődi, F., Csontos, C.:
Characterization of the effect of TIMAP phosphorylation on its interaction with protein
phosphatase 1.
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DOI: <http://dx.doi.org/10.1002/cyto.a.20347>
IF:3.293

Total IF: 15.696

Total IF (publications related to the dissertation): 5.858

The Candidate's publication data submitted to the Publication Database of the University of Debrecen
have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and
Journal Citation Report (Impact Factor) databases.

01 August, 2013



12. KEYWORDS

Endothelium

Barrier function

Protein phosphatase 2A

Adherens junctions

β -catenin

CPI-17

Plakoglobin

Immunofluorescence

Transendothelial resistance measurement

Co-immunoprecipitation

13. TÁRGYSZAVAK

Endotélium

Barrier funkció

Protein foszfatáz 2A

Adherens sejt kapcsolatok

β -catenin

CPI-17

Plakoglobin

Immunfluoreszcencia

Transzendotél ellenállás mérés

Ko-immunprecipitáció

14. ACKNOWLEDGEMENT

I wish to express my sincere gratitude to my supervisor Dr. Csilla Csontos for helping and guiding my work.

I sincerely thank Prof. Pál Gergely former and Prof. Virág László present head of Department of Medical Chemistry for giving all the support for my work.

I also wish to express my gratitude to all the members of the Department of Medical Chemistry. My special thanks to Dr. István Czikora, Dr. László Kovács, Dr. Brunyánszki Attila and Róbert Bátori who rendered their help during my work.

Special acknowledgements must be given to Ms. Ella Kovács, Ms. Zsanett Molnár, Ms. Andrea Farkas, Ms. Ágota Szántó and Ms. Julianna Hunyadi for the excellent technical assistance.

A special thanks to György Vámosi for his help in the confocal microscopy.

Additionally, I am thankful to our collaborator Prof. Alexander D. Verin, who gave me the opportunity to work and gain experience in his lab, at the Georgia Regents University.

I would also like to acknowledge my family for supporting me.

I am also grateful to my husband for helping and encouraging me.

15. APPENDIX

Presentations 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

39th. Membrane-Transport Conference 2009, Sümeg

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, Hungary, August 23-26,

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

Hungarian Biochemical Society, Annual Meeting 2010, Budapest

6. István Czikora, Kyung-mi Kim, **Anita Kása**, Bálint Bécsi, Alexander D. Verin, Pál Gergely, Ferenc Erdődi, Csilla Csontos: Characterization of the effect of TIMAP phosphorylation on its interaction with protein phosphatase 1

Europhosphatases 2011, Baden near Vienna, Austria, 18 - 23 July, 2011

7. **Anita Kása**, Pál Gergely, Csilla Csontos: The role of Protein phosphatase 2A in the regulation of β -catenin dephosphorylation in ECs

Hungarian Biochemical Society, Annual Meeting 2011, Pécs

8. **Anita Kása**, Csilla Csontos, Pál Gergely, and Alexander D. Verin: B α regulatory subunit targets PP2A to Adherens Junctions and regulates the dephosphorylation of β -catenin in endothelial cells
American Heart Association, Scientific Sessions 2012, Los Angeles
9. **Anita Kása**, Pál Gergely, Alexander D. Verin, Csilla Csontos: Phosphatase 2A is involved in Adherens Junction Regulation in Endothelial Cells
Experimental Biology 2013, Boston
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
American Thoracic Society, 2013, Philadelphia