Ph.D. thesis

THE ROLE OF PROTEIN PHOSPHATASE 2A (PP2A) IN THE REGULATION OF PULMONARY ARTERY ENDOTHELIAL CELL CYTOSKELETON STRUCTURE

KRISZTINA TAR

Supervisor: Csilla Csortos, Ph.D
associate professor

UNIVERSITY OF DEBRECEN
MEDICAL & HEALTH SCIENCE CENTER FACULTY OF MEDICINE,
DEPARTMENT OF MEDICAL CHEMISTRY
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INTRODUCTION

Ser/Thr and Tyr protein phosphorylation/dephosphorylation

Protein phosphorylation/dephosphorylation is a principal regulatory mechanism in the control of almost all cellular processes. Based on molecular biology data the eurokaryotic protein phosphatases are structuraly and functionally diverse enzymes that are represented by three distinct gene families. Two of these, the PPP and PPM families, dephosphorylate phosphoserine and phosphothreonine residues, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine amino acids. A subfamily of the PTPs, the dual-specificity phosphatases, dephosphorylate all three phosphoamino acids. Within each family, the catalytic domains are highly conserved, with functional diversity endowed by regulatory domains and subunits. The Ser/Thr protein phosphatases are metalloenzymes and dephosphorylate their substrates in a single reaction step using a metal-activated nucleophilic water molecule. In contrast, the PTPs catalyze dephosphorylation by use of a cysteinyl-phosphate enzyme intermediate. Ser/Thr protein phosphatases can be conveniently divided into two classes, type-1 and type-2. Type-1 protein phosphatases dephosphorylate the beta-subunit of phosphorylase kinase and are potently inhibited by two thermostable proteins termed inhibitor-1 and inhibitor-2, whereas type-2 protein phosphatases preferentially dephosphorylate the alpha-subunit of phosphorylase kinase and are insensitive to inhibitor-1 and inhibitor-2. The substrate specificities of the four enzymes, namely protein phosphatase-1 (type-1) and protein phosphatases 2A, 2B and 2C (type-2) have been investigated. Protein phosphatase-2C is an Mg^{2+}-dependent enzyme, protein phosphatase-2B is a Ca^{2+}-calmodulin-dependent enzyme, while PP2A does not require metal ion for its activity, and it can be inhibited by okadaic acid. In the regulation of endothelial cell barrier function, the role of PP1 and PP2B was observed and it is known that both enzyme is involved in the

maintenance of endothelial cell cytoskeleton and barrier function. The exact role of PP2A in the regulation of EC cytoskeleton is virtually unexplored.

Protein phosphatase 2A

PP2A is a highly conserved Ser/Thr specific protein phosphatase. Several holoenzyme complexes of PP2A have been isolated from a variety of tissues and have been extensively characterized. The core enzyme is a dimer ($PP2A_D$), consisting of a 36kDa catalytic subunit ($PP2A_C$) and a regulatory subunit of molecular mass 65kDa, termed PR65 or the A subunit. A third regulatory B subunit can be associated with this core structure. Molecular cloning revealed the existence of two mammalian $PP2A_C$ isoforms, α and β, which share 97% identity in their primary sequence. Both isoforms are ubiquitously expressed, and very high levels are found in brain and heart. However, $PP2A_{C\alpha}$ is about 10 times more abundant than $PP2A_{C\beta}$. Molecular cloning of $PP2A_C$ from lower organisms, has revealed that the structure of $PP2A_C$ has remained remarkably constant throughout evolution, and may even be the most conserved of all known enzymes. The A subunit is a structural subunit that is tightly associated with $PP2A_C$, forming a scaffold to which the appropriate B subunit can bind. As is the case for the catalytic subunit, in mammals two distinct PR65 isoforms are present, α and β, which share 86% sequence identity. The PR65 is entirely composed of 15 tandem repeats of a 39 amino-acid sequence, termed a HEAT (huntingtin/elongation/A subunit/TOR, where TOR is target of rapamycin) motif. Tandem repeats of HEAT motifs are found in a variety of proteins, including the huntingtin protein, an elongation factor required for protein synthesis and the TOR kinase. The crystal structure of PR65α revealed that the fundamental architecture of each repeat is virtually the same, being composed of two superimposed α-helices. The particular stacking of these repeats within the PR65 molecule gives rise to a stable protein with an overall

asymmetrical and elongated architecture, reminiscent of a hook (C-shape). The catalytic and the different B subunits bind to the A subunit at well established portions of the protein. The A subunit binds the catalytic subunit through repeats 11 to 15 at the C terminus and the tumor antigens encoded by small DNA tumor viruses through overlapping but distinct regions at N-terminal repeats 2 to 8. The binding region for the B subunit to include repeats 2 to 8, covers repeats 1 to 10, and the B and C subunits cooperate in binding to the A subunit. The third component of the heterotrimer is the B subunit. The different heterotrimeric PPase 2A enzymes diverse functionally by means of the B subunit. There are four unrelated gene families coding several isoforms and splice variants of the B subunits, termed B, B', B", B"'. The structures of B, B', B", B"' are completely different; it seems that they direct the enzyme to multiple cellular function, and distinct subcellular compartments.

Biological role of PP2A

It has long been known that the essential role of PP2A is the dephosphorylation of proteins. The diversity of PP2A caused by the regulatory B subunit suggests the participation of PP2A in many biological processes such as cell cycle, signal transduction, DNA replication, cell transformation or apoptosis. At present, one of the best-documented cyclin-dependent kinase (Cdk)–cyclin complexes is MPF (M-phase-promoting factor), which consists of the $p34^{cdc2}$ kinase (Cdc2 or Cdk1) and cyclin B, and is involved in the G_2/M transition. MPF is essential during G_2/M transition. PP2A is required to maintain MPF in its inactive precursor form. INH, originally identified as an activity that could inhibit activation of pre-MPF, was indeed shown to be a form of PP2A, more particularly a trimer containing the Bα/PR55 subunit. PP2A was identified also as an interaction partner of Cdc6, a protein required for the initiation of DNA replication. PR48 localizes to the nucleus, and Cdc6 seems to be a selective substrate for the PR48-containing

PP2A trimer. As with PR59, overexpression of PR48 causes a G_1 arrest. It is believed that PP2A keeps Cdc6 in the dephosphorylated form, a prerequisite for binding to origins of DNA replication.

PP2A is also an important cellular target for viral antigens such as polyoma small t and middle T, as well as SV40 small t, form stable complexes with $PP2A_D$ by displacing the third subunit and affecting cell transformation. PP2A is the only cellular protein known to bind to SV40 small t. In addition to its role the HIV-1-encoded protein vpr has been shown to mediate a G_2 arrest through a specific interaction with B/PR55, targeting the complex to the nucleus and leading to dephosphorylation of Cdc25.

The dephosphorylation of HSPs is largely unexplored. A novel interaction between heat shock transcription factor 2 (HSF2) and the PR65/A subunit of PP2A was described and showed that HSF2 is able to compete with the PP2Ac for binding to PR65. Others suggested that PP2A can dephosphorylate HSP27 *in vitro,* suggesting that PP2A is the key enzyme dephosphorylating HSP27 in the cells.

Two-hybrid assays have indicated that the B´/PR61α and B´/PR61δ subunits of PP2A can associate with cyclin G, indicating the the role of PP2A in DNA degradation.

PP2A can modulate the activities of several kinases *in vitro* and *in vivo*, in particular phosphorylase kinase, the ERK/MAPKs, the calmodulin-dependent kinases, PKA, protein kinase B (PKB), PKC.

Endothelial cell cytoskeleton and barrier function

Endothelial cells (EC) form a confluent monolayer on the surface of the inner wall of blood vessels and participate in the regulation of many physiological and pathological processes. One of their major functions is the separation of blood from underlying tissues, allowing only tightly controlled passage of macromolecules and cells. Specialized transcellular systems of transport vesicles and the coordinated opening and closure of cell-cell junctions participate in maintaining endothelial integrity, which is vital for the protection

of vessels from any uncontrolled increase in permeability or inflammation. Intercellular gap formation evoked by bioactive agents such as thrombin, results in increased endothelial permeability, a typical feature of acute inflammatory lung syndrome. Coordinated functioning of the components of the cytoskeleton governs gap formation and EC barrier integrity. Reversible phosphorylation of numerous cytoskeletal proteins has crucial role in the maintenance of appropriate alignment of the cytoskeleton. It is well established that acto-myosin interaction and cell contraction is critically dependent upon the level of myosin light chains (MLC) phosphorylation. Increased MLC phosphorylation on Ser19, initiates F-actin stress fiber formation, which leads to paracellular gap formation and finally to EC barrier dysfunction. MLC phosphorylation level is determined by the balanced activities of MLC kinase (210 kDa) and MLC PPase, which belongs to type 1 family of Ser/Thr protein phosphatases. The 130 kDa myosin binding subunit of myosin phosphatase can be phosphorylated by Rho-kinase, and its activity is inhibited by this phosphorylation reaction. Another possible regulator of MLC PPase is a recently discovered endogenous protein inhibitor CPI17 (for PKC-potentiated inhibitory protein of 17 kDa) in smooth muscle. PP2A can also dephosphorylate CPI17 *in vitro* in smooth muscle extracts, and CPI17 was described in endothelial cells.

Protein phosphatase 2A and the cytoskeleton

The role of PP2A in the barrier function of endothelial cells is largely unexplored. However, recent data support its involvement in the regulation of several cytoskeletal and cytoskeletal-associated proteins (tau, vimentin, β_{III}-tubulin, HSP-27, caldesmon), but cytoskeletal targets of PP2A are not well characterized.

PP2A is recognized as a protein phosphatase interacting with the microtubules (MT) and MT-associated proteins (MAPs). PP2A is co-localized with microtubules and MAPs such as tau protein, though

information is limited about the linkage between the microtubule network and contractile cytoskeleton. Several proteins which bind to microtubules and microfilaments have been identified. They include microtubule-associated proteins (MAP1B, MAP2, tau) and the regulatory cytoskeletal protein, caldesmon It has became well established that microtubule-associated proteins (MAPs) control microtubule polymerization/depolymerization and are critically involved in the ability of microtubules to respond to diverse signals such as those that induce cell division and cell motility. Dephosphorylation of hyperphosphorylated tau by PP2A and PP2B restores its ability to promote microtubule assembly. Thus, biological function of tau is regulated by phosphorylation.

PP2A can associate not only with MAPs, but other cytoskeletal proteins and it is able to dephosphorylate those proteins. For instance in neuroblastoma cells vimentin has been shown to be phosphorylated in vitro and/or in vivo by various protein kinases, including Rho-kinase, and reversible phosphorylation has been shown to play a key role in their disassembly. Dephosphorylation by PP2A makes the process reversible. The intermediate filament protein vimentin is a major phosphoprotein in mammalian fibroblasts, and reversible phosphorylation plays a key role in its dynamic rearrangement. Selective inhibition of type 2A but not type 1 protein phosphatases led to hyperphosphorylation and concomitant disassembly of vimentin, characterized by a collapse into bundles around the nucleus. Also the association of vimentin was shown with PP2Ac, A and B/PR55 subunits.

Cofilin, an actin-depolymerizing protein, is essential for the functional dynamics of the actin cytoskeleton and for cell viability. In unstimulated human peripheral blood T lymphocytes cofilin is phosphorylated and localized in the cytoplasm. Following co-stimulation through accessory receptors (e.g. CD2 or CD28) cofilin undergoes dephosphorylation. The subcellular localization as well as the actin-binding activity of cofilin are regulated by the phosphorylation state of serine-3. Thus, only the dephosphorylated form of

cofilin associates with the actin cytoskeleton and possesses the capability to translocate into the nucleus The serine/threonine phosphatases of type 1 and type 2A not only associate with cofilin but also dephosphorylate this 19-kDa protein and thereby mediate cofilin activation.

HSP27 is a terminal substrate of the stress-activated p38 MAPK cascade. The ability of PP2A to dephosphorylate HSP27 is shown to be regulated by the phosphorylation state of PP2A itself. The phosphorylation state of HSP27 determines its effect on actin polymerization. Non-phosphorylated small HSP monomers are active in inhibiting actin polymerization while phosphorylated monomers and non-phosphorylated oligomeric forms are inactive.

It was documented, that microfilaments and microtubules both has an important role in maintaining cytoskeletal structure in endothelial barrier function. It is also suggested that PP2A can be critical in dephosphorylating possible substrates involved in regulation of barrier function and regulation of cytoskeletal structure through microtubules and microfilaments.

AIMS

Our goal was to study the linkage among the PP2A, the barrier function and the regulation of cytoskeleton in EC. To clarify the role of PP2A in EC, we focused on the next problems:

1. To study the effect of PP2A inhibition on the endothel monolayer and on cytoskeletal acto-myosin structure.

2. To examine the localization of PP2A in EC.

3. To study the effect of PP2A inhibition on PP2A localization and on microtubule structure.

4. To prepare mammalian expression vectors and adenoviral system to introduce PP2A subunits into HPAEC and into BPAEC.

5. Localization of the overexpressed PP2A subunits; to examine the effect of the overexpressed proteins on actin, on microtubules and on barrier function in EC.

6. Inhibition of the overexpressed PP2A subunits and to determine the effect of the inhibition on EC actin cytoskeleton and on barrier function.

7. To study the effect of the overexpressed PP2A subunits on the phosphorylation level of substrate candidates, such as tau and HSP27.

We intended to clarify the involvement of PP2A-mediated tau and HSP27 dephosphorylation in alteration of EC structure and to highlight the potential importance of PP2A in the regulation of endothelial cell cytoskeleton and barrier function.

METHODS

Cell cultures Bovine Pulmonary Artery Endothelial Cells (BPAEC) were utilized at passages 15-24. Cells were maintained in Medium 199 (GIBCO BRL), supplemented with 20% (v/v) colostrums-free bovine serum (Irvine Scientific), 15 μg/ml endothelial cell growth supplement (Collaborative Research), 1% antibiotic and antimycotic solution (penicillin, 10,000 units/ml; streptomycin, 10 μg/ml; and amphotericin B 25 μg/ml; K. C. Biologicals), and 0.1 mM non-essential amino acids (Gibco). Human Pulmonary Artery Endothelial Cells (HPAEC), human umbilical vein EC (HUVEC), and human lung microvascular EC (HLMVEC) were obtained from Clonetics, BioWhittaker Inc. and were cultured in endothelial basal medium (EBM)-2 growth media supplemented with 0.2 ml of hydrocortisone, 2 ml of human FGF-B, 0.5 ml of VEGF, 0.5 ml of long-arm insulin-like growth factor-1 (R^3-IGF-1), 0.5 ml of ascorbic acid, 0.5 ml of human epidermal growth factor (EGF), 0.5 ml of GA-1000, and 0.5 ml of heparin (Clonetics)/500 ml with 10% FBS. All cells were maintained at 37 ^oC in a humidified atmosphere of 5% CO_2 and 95% air. HPAEC were utilized at passages 6–10, HUVEC at passages 2–3, and HLMVEC at passages 4–8. AD-293 cell line was obtained from Stratagene. AD-293 cells were derived from the commonly used HEK293 cell line, but have improved cell adherence and plaque formation properties. Cells were cultured in DMEM (containing 4.5 g/L glucose and 110 mg/L sodium pyruvate and 2 mM L-glutamine), supplemented with 10% heat-inactivated fetal bovine serum. Cells were maintained at 37 ^oC in a humidified atmosphere of 5% CO_2 and 95% air.

HUVEC cDNA library screening 10^6 clones of a random hexamer and oligo dT primed HUVEC λgt 11 cDNA library (kindly provided by Dr. David Ginsburg, Ann Arbor, USA) were screened. The RT-PCR amplified human liver PP2Ac, and the alfalfa PP2Aa subunit homologue (kindly provided by Dr. Viktor Dombrádi, University of Debrecen, Hungary) DNA probes were random primed labeled with ^{32}P, and hybridization was carried out (1-2x10^6 cpm/ml

hybridization buffer) at 55 ^{o}C with the immobilized clones. Labeled DNA probes bound non-specifically to the membrane were removed by washing with 2XSSC at 60 ^{o}C. Positive signals were detected by autoradiography. After at least three cycles of subsequent screening of the library the largest identified clones were tested by sequencing and the full length cDNAs for PP2Ac and PP2Aa were isolated/constructed using standard cloning methodologies.

Mammalian expression plasmids The entire coding sequence of PP2Ac (930 bp), PP2Aa (1770 bp) and PP2Aa$_N$ (aa 1-395, 1185 bp) were amplified by PCR using the isolated HUVEC lambda phage clones as template. The PCR products were subcloned into the mammalian pCMV-HA (PP2Ac) and pcDNA3.1/*Myc*-His (+) A version (PP2Aa and PP2Aa$_N$) expression vector. All constructs and their open reading frames were analyzed by sequencing using vector and PP2A specific primers.

Transfection Cells were grown to 70 % of confluency, incubated with the appropriate PP2A subunit (PP2Ac, PP2Aa, PP2Aa$_N$) construct in the presence of FuGene 6 transfection reagent (Roche), according to manufacturer's protocol. After 24 or 48 hours of incubation (37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air) the cells were washed 3x with 1x PBS and used for further experiments.

In vitro phoshatase activity assay After transfection the cells were washed three times with 1X PBS, then lysed in lysis buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, 28 mM 2-ME, 0.5 mM PMSF, 2 mM benzamidine). To measure the phosphatase activity the samples were diluted 1:20 with buffer containing 20 mM Tris-HCl (pH 7.4), 0.1% 2-ME and 1mg/ml BSA. Since MLC is an *in vitro* substrate for both PP1 and PP2A, we used 5 nM okadaic acid, specific inhibitor of PP2A at this concentration, to differentiate between PP1 and PP2A activities. ^{32}P-labeled MLC were prepared by phosphorylation of 2mg/ml MLC (MLC substrate was obtained courtesy of Dr. Ferenc Erdődi, University of Debrecen, Hungary) with 10 μg/ml MLCK in the presence of 15

μg/ml calmodulin, 0.2 mM $CaCl_2$, 0.25 mM [γ–^{32}P]ATP (600-1,000 counts.min^{-1}.pmol^{-1}) and 5 mM Mg-acetate at 30 ^{o}C for 5 min. ^{32}P-MLC (~ 1 mol phosphate/mol MLC) was extensively dialyzed to remove the excess of ^{32}P-ATP. The reaction was started by the addition of the substrate, and the released ^{32}P was determined from 400 ml of supernatant according to the method of Erdodi at al.

Adenoviral plasmids The entire coding sequences of PP2Ac and PP2Aa were subcloned into pShuttle-Ires-hrGFP-2-HA vector. The entire coding sequences of the recombinants were verified by sequencing. Adenoviral recombinant plasmids were prepared with homologous recombination of the above plasmids with pAdEasy-1 viral DNA plasmid according to manufacturer's protocol (Stratagene), and were verified by restriction analysis. As control, pShuttle-CMV-lacZ-pAdEasy-1 was used.

Viral preparation Virus was prepared according to the published methods (Hee et al., 1997) and manufacturer's protocol (Stratagene) with slight modifications. Final viral stocks produced were in 10^{10}-10^{11}pfu/ml range.

Viral infection Serial dilutions of the purified virus were prepared in serum free medium. The media was aspirated from the plates of interest and replaced with the diluted virus. Complete medium containing serum and growth supplements were added 1 hour later (see: Materials and Methods/cell culture). After overnight incubation the medium was changed again to complete medium and after 24 hours of incubation, infected cells were used in further experiments. Transfected/infected cells containing the HA- and the GFP-tag were tested by Western blot analysis or immunostaining procedure.

Measurement of Electrical Resistance across Endothelial cell Monolayers Endothelial cells were grown to 90% or 100% confluence on gold microelectrodes in polycarbonate wells. Transmonolayer electrical resistance (TER) was measured using an electrical cell-substrate impedance sensing system (ECIS, Applied Biophysics Inc.), indicating the state of cell

shape, cell-cell contact as well as cell adhesion to extracellular matrix. 30-60 min after starting the measurement, agonists were added to wells. Data were collected for periods of 5 hr.

EC fractionation EC fractionation was performed using two different fractionation protocols. Initial protocol allows to separate 3 cellular fractions, which include microtubule-enriched, cytoskeletal (detergent insoluble F-actin-enriched), and cytosolic fractions. Second (more advanced, but more complex) protocol allows to separate cytosolic, tubulin, actin and intermediate filaments (vimentin)-enriched fractions.

Western immunoblotting Cells for Western immunoblotting after transfection or infection protocols were washed 3x with 1x PBS then scraped from the dishes and lysed with 2x boiling SDS sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0,006% bromophenol blue, 2% 2-mercaptoethanol), then passed through a 26 gauge needle. After 5 minutes boiling, the protein samples were centrifuged for 2 minutes and separated by SDS-PAGE on 10% gels. Next the proteins were transferred to nitrocellulose membrane, and incubated with specific antibodies of interest for 120-180 min. Immunoreactive proteins were detected with enhanced chemiluminescent detection system (ECL) according to the manufacturer's directions (Amersham).

Immunofluorescent staining After specific treatments, EC grown on glass coverslips were fixed in 3.7% formaldehyde solution in PBS for 10 min at 4 ^{o}C, washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBST for 30 min at room temperature, and blocked with 2% BSA in PBST for 30 min. PBS/PBST was replaced by TBS/TBST when phospho-specific primary antibodies were employed. Incubation with antibodies was performed in blocking solution for 1 hour at room temperature. Alexa 350-, 488 -, Alexa 594-conjugated secondary antibodies were used for immunodetection. Actin filaments were stained with Texas Red-phalloidin. After immunostaining procedures slides were analyzed using a Nikon video-imaging system

consisting of a phase contrast inverted microscope connected to a digital camera and image processor. The images were recorded and processed using Adobe Photoshop program.

RESULTS AND DISCUSSION

A major function of the vascular endothelial cell (EC) monolayer is to serve as a selective barrier to fluid and solute flux across the blood vessel wall. Increased endothelial permeability is a prominent characteristic of acute inflammatory lung syndromes and is the result of intercellular gap formation evoked by bioactive agents. EC barrier integrity is administrated by a dynamic equilibrium between competing contractile and tethering forces. The equilibrium is dependent on the coordinated functioning of the components of the cytoskeleton and is regulated via reversible phosphorylation of numerous cytoskeletal proteins. Acto-myosin interaction and cell contractility depends upon the phosphorylation state of myosin light chains (MLC). Increased level of MLC phosphorylation is followed by actin redistribution, filament formation, and EC barrier dysfunction. Beside the physical interaction due to direct or indirect protein-protein interactions among the three major elements of the cytoskeleton, microfilaments, microtubules and intermediate filaments, recent results suggest that direct linkage between microtubules and microfilaments is important for coordinated signaling, which affect changes in EC cytoskeletal architecture.

The focus of the present study was to determine the role of PP2A in EC cytoskeleton, and identify its cytoskeletal protein targets involved in endothelial barrier function using pharmacological inhibitors and thrombin. Nocodazole is a synthetic antitubulin agent that reversibly blocks the assembly of tubulin and depolymerizes preformed microtubules. Okadaic acid is a polyether derivative of 38-carbon fatty acid which implicated as the causative agent of diarrheic shellfish poisoning and also a powerful inhibitor of type 1 and 2A protein phosphatases. Okadaic acid inhibits the phosphatases to different extents *in vitro*: PP2A is inhibited more strongly (K_i=0.2 nM) compared to PP1 (K_i=2 μM). Okadaic acid up to 1 μM does not have any detectable effect on the major Ser/Thr phosphatase activities in

living cells except PP2A, and can therefore, be utilized for studying PP2A function.

It was previously shown that okadaic acid at 2-5 nM concentration had no significant effect on bovine EC permeability and MLC phosphorylation, suggesting a limited role of PP2A alone in direct regulation of EC contractility and barrier function. It was also shown that okadaic acid even up to 100 nM concentration inhibited only PP2A in intact BPAEC. On the other hand, it was shown, that microtubule destabilization by nocodazole (2-5 μM) significantly increased EC actomyosin contraction and permeability indicating the importance of microtubules in maintaining the EC barrier.

Since PP2A was reported to bind and dephosphorylate several MAPs like tau, which may affect microtubule structure, we examined the role of PP2A in microtubule stability and indirectly in nocodazole-induced contractile responses and permeability in EC. Our data indicated that okadaic acid (5 nM) significantly potentiated the effect of suboptimal doses of nocodazole (50-200 nM) on TER and formation of paracellular gaps. These results suggested the participation of PP2A activity in the cross-talk between actin-cytoskeleton and microtubules. To confirm association of PP2A with microtubules in EC we applied two different methods of subcellular fractionation combined with Western blot analysis. By both methods we detected the majority of PP2A in the tubulin/microtubule-enriched fractions. The occurrence/distribution of PP2A protein or activity was studied by others in many subcellular fractions which were prepared and studied by different methods. For instance the activities and concentrations of protein phosphatase type 1 (PP1) and type 2A (PP2A) were compared in cytosol and particulate fractions of rat forebrain. Although the activity of PP2A was highest in the cytosol, immunoblot analysis with a PP2A-specific antibody showed that there were significant levels of the enzyme in the particulate fraction. There was no significant difference between the concentration of PP2A in the cytosol and particulate fractions such that the low activity of

PP2A in the particulate fraction represents an inactivation of this form of the enzyme Association of PP2A and microtubules was also described by others based on fractionation, and Western immunoblotting analysis in rat or bovine brain tissues. Immunofluorescence microscopy revealed the presence of protein phosphatase 2A (PP2A) on microtubules in neuronal and nonneuronal cells. Interphase and mitotic spindle microtubules, as well as centrosomes, were all labeled with antibodies against individual PP2A subunits, showing that the AB alpha C holoenzyme is associated with microtubules. Also it was shown that protein phosphatase (PP) 2A1, a trimer composed of A-, B- and C-subunits in the PP2A family, has been regarded as a principal form localizing at microtubules (MT), but PP2A2, the dimer of A- and C-subunits, has not. PP2A1 but not PP2A2, both isolated from bovine extract, largely associated with the purified preparation of MT. Furthermore, PP2A1 was found to bind purified tubulin polymerized by taxol. The presence of MT associated proteins with purified tubulin hardly affected the binding of PP2A1 to the tubulin.

Our immunofluorescent staining of EC with PP2A antibody revealed microtubule-like pattern in quiescent cells. This pattern is completely disappeared after treatment of cells with okadaic acid. Changes in PP2A subcellular localization after PP2A inhibition co-incide with disappearance of peripheral microtubules. Taken together these results suggested that inhibition of PP2A phosphatase activity caused dissociation of PP2A from microtubules and destabilization of microtubule network.

To further clarify the role of PP2A in the regulation of EC cytoskeleton structure we cloned and prepared mammalian expression plasmids of the catalytic C subunit (PP2Ac), the regulatory A subunit (PP2Aa), and its truncated mutant ($PP2Aa_N$). HPAEC was transfected with the recombinant expression plasmids. Previous reports indicated problematic overexpression of PP2Ac, since it is a tightly regulated protein. However, functional expression of PP2Ac was achieved with N-terminal HA-tag. Therefore we

performed transient expressions with 24 h post-transfection time; and for the expression of PP2Ac we also utilized an expression vector providing N-terminal HA-tag. The activity of the expressed PP2A was demonstrated by *in vitro* phosphatase assays and by specific inhibition of the enzyme by okadaic acid. Endothelial cells have low transfection efficiency, and that was our observation too. Therefore, although according to the immunostaining pictures the recombinant protein were present in high concentration in those cells which expressed the recombinant, we detected only about 30-50 % increase in PP2A activity compared to appropriate controls. The cells containing either the recombinant PP2Ac alone or recombinant PP2Aa and PP2Ac looked healthy and did not differ from the neighboring (control) cells in term of shape, suggesting that the catalytic subunit was likely properly associated with native regulatory proteins; or recombinant A subunit, which could bind native B-subunit(s) and control the PP2A activity.

PP2Ac overexpression and co-expression of PP2Ac and PP2Aa rearranged the actin cytoskeleton as we demonstrated by immunofluorescent staining; it led to dramatic decrease of stress fibers in the cells and the peripheral actin region became thicker. Both PP2Ac and PP2Aa subunits seemed to co-localize with F-actin suggesting protein-protein interactions between PP2A and actin or actin-binding protein(s). Association of PP2A with actin and other cytoskeletal proteins were also found in epithelial cells of lung and kidney.

The A subunit serves as a coordinator to assemble the catalytic C subunit and one of the B subunits of PP2A. The structure of the A subunit is quite well understood, the catalytic and the different B subunits bind to the A subunit at well established portions of the protein. We generated and overexpressed a truncated form of the A subunit, $PP2A_N$. This mutant contains the N-terminal portion of PP2Aa, the region which is assigned to bind the B-subunits, but does not have the C-terminal protein sequence capable to bind PP2Ac. Interestingly, PP2Ac co-expressed with the mutant $PP2A_N$ seems not to exhibit any catalytic activity on actin/or actin-binding proteins, based on the

results of immunostaining, suggesting that association of PP2Ac with PP2Aa+b is critical to target the PP2Ac to its appropriate substrates. In agreement with this speculation, recent publication of Strack et al. (2004) presents data indicating the critical role of PP2A heterotrimers in mammalian cell survival. They generated stable PC12 cell lines in which the major scaffolding Aalpha subunit can be knocked down by inducible RNA interference (RNAi) to study its role in cell viability. Aalpha RNAi decreased total PP2A activity as well as protein levels of C, B, and B'. Other experiments indicate that monomeric C and B subunits are degraded by the proteosome.

Verin et al., (2001) have shown that microtubule disassembly significantly increases EC actomyosin contraction and permeability indicating that like microfilaments, microtubules also play crucial role in maintaining the EC barrier They have previously shown that protein phosphatase 1 (PP1) associates with myosin filaments and is directly involved in EC contractility and barrier regulation through the dephosphorylation of MLC. In addition, they have cloned and characterized several isoforms of the endothelial MLC kinase (MLCK) which are the major known influencing level of MLC phosphorylation. Although the events at molecular level linking the cytoskeletal changes initiated by microtubule disruption and cell contraction are not clarified yet, they demonstrated a correlation between microtubule disruption, activation of the contractile machinery (MLC phosphorylation, stress fiber formation), and barrier dysfunction. The increased level in MLC phosphorylation after microtubule disruption in ECs suggests activation of biochemical signal pathways that lead to contraction and EC barrier failure.

Although it is well-accepted that PP2A associates with MT/MAP, PP2A overexpression did not significantly affect MT structure of quiescent EC but definitely preserved MT network in nocodazole-treated cells.

It was previously shown, that both MT-inhibitor nocodazole and receptor-mediated edemagenic agonist, thrombin, induced a profound EC barrier dysfunction via rearrangement of F-actin cytoskeleton.

Overexpression of active PP2A prevented or considerably reduced the effect of these agents on the F-actin. Taken together, these data for the first time demonstrated the critical role of PP2A in maintaining EC cytoskeletal structure.

In order to directly examine the effect of PP2A on EC permeability, we generated recombinant adenoviral constructs for PP2Aa and PP2Ac subunits. As we demonstrated by Western blot analysis and immunofluorescent staining with specific antibodies, we successfully infected EC with these recombinant adenoviral constructs with approximately 100% transfection efficiency. Infection of HPAEC with PP2Aa+c significantly attenuated thrombin-, or nocodazole-induced decrease in transendothelial electrical resistance. These novel data also indicated that PP2A is directly involved in the EC barrier protection most likely via dephosphorylation of specific cytoskeletal targets, which results in the preservation of EC cytoskeleton.

To find physiological substrates for PP2A in EC cytoskeleton, we focused on two cytoskeletal regulatory proteins, HSP27 and tau. We hypotised that the dephosphorylation of the two regulatory proteins by PP2A can link the regulation of microtubules and actin. We demonstrated that significant amounts of tau, and HSP27 along with PP2A were present in the microtubule-enriched EC fraction suggesting the tight association of these proteins with microtubules in endothelium, and the possible role of PP2A in the dephosphorylation of tau and HSP27. The association of HSP27 with tubulin/microtubules in HeLa cells was reported. HSP27, depending on its phosphorylation state, affects actin stress fiber and focal adhesion formation, two important components in EC barrier regulation and it has been also recognised as a potent regulator of cytoskeletal dynamics. Several studies have shown that overexpression of HSP27 increases the stability of F-actin microfilaments during exposure to such stresses as hyperthermia, oxidants and cytochalasin D, but the exact mechanism is poorly understood. However, the phosphorylation state of HSP27 determines its effect on actin

polymerization. Non-phosphorylated small HSP monomers are active in inhibiting actin polymerization while phosphorylated monomers and non-phosphorylated oligomeric forms are inactive. The majority of published data indicated that under non-stimulating conditions HSP27 is diffusely distributed in the cytoplasm, although according to other publications it is co-localized with actin. Recent *in vivo* results also suggest correlation between endothelial barrier dysfunction evoked by lipopolysaccharide with phosphorylation of HSP27.

Our Western blot analysis and immunoflourescent staining show that the treatment of EC with nocodazole led to significant increase in HSP27 phoshorylation in nontransfected cells, while PP2A subunits overexpression significantly decreased the level of nocodazole-induced HSP27 phosphorylation, which correlates with the protective effect of PP2A on EC barrier. Nocodazole-induced MT dissolution increased F-actin stress fiber formation only in the control, but not in the PP2A-transfected cells indicating the involvement of PP2A activity in the dynamic cross-talk between the actin cytoskeleton and MT.

Microtubule-associated protein, tau, in its unphosphorylated form promotes assembly of microtubules and inhibits depolymerization. Phosphorylation of tau by several kinases, including Ca^{2+}/calmodulin-dependent kinase II and protein kinase A, decreases its capability to bind microtubules as well as to promote microtubule assembly. On the other hand, dephosphorylation of hyperphosphorylated tau restores its ability to promote microtubule assembly. Thus, biological function of tau is regulated by phosphorylation. Galpha(12/13) interact with several cytoskeletal/scaffolding proteins, and in a yeast two-hybrid screen with Galpha(12), an interaction was detected with the scaffolding subunit (Aalpha) of the Ser/Thr phosphatase, protein phosphatase 2A (PP2A). PP2A dephosphorylates multiple substrates including tau, a microtubule-associated protein that is hyperphosphorylated in neurofibrillary tangles. The interaction of Aalpha and

Galpha(12) was confirmed by coimmunoprecipitation studies in transfected COS cells and by glutathione S-transferase (GST)-Galpha(12) pull-downs from cell lysates of primary neurons. These findings reveal novel, direct regulation of PP2A activity by Galpha(12) and potential in vivo modulation of PP2A target proteins including tau.

Our results show that the treatment of EC with PP2A inhibitor, okadaic acid, led to significant increase in tau phosphorylation confirming that tau is a substrate of PP2A in endothelium. Simultaneous inhibition of PP1 and PP2A by calyculin did not significantly increase the level of tau phosphorylation compared to inhibition of PP2A alone suggesting a limited role of PP1 activity in tau dephosphorylation. For anti-phospho tau staining we utilized an antibody specific for P-Ser262, the phosphorylation site with the strongest impact on binding capacity of tau to microtubules. Inhibition of PP2A led to translocation of phospho-tau to cell periphery, and phosphorylated tau was preferentially localized in the cytosolic fraction. Accordingly, we detected disassembly of peripheral microtubules in the okadaic acid-treated EC. These data suggested the direct link between PP2A-mediated tau dephosphorylation and stability of peripheral microtubules in EC. Microtubule-associated protein, tau, is an *in vitro* substrate for a number of protein kinases including p38 MAP kinase. Tau has several isoforms, formed by alternative mRNA splicing of a single gene. Tau is predominantly found in neuronal cells but has been reported in several non-neuronal cells including fibroblasts and lymphocytes. Our data indicate that PP2A overexpression significantly decreased tau phosphorylation induced by nocodazole, which correlates with attenuation of nocodazole-induced cytoskeleton rearrangement and permeability. Collectively, these results suggested that both tau and HSP27 may be physiological cytoskeletal substrates of PP2A in endothelium, which may be involved in PP2A-mediated EC barrier protection.

SUMMARY

The focus of the present study was to determine the role of PP2A in human and bovine pulmonary artery endothelial cell cytoskeleton regulation, and identify its cytoskeletal protein targets involved in endothelial barrier function We have shown that okadaic acid (2-5 nM), a powerful inhibitor of protein phosphatase 2A (PP2A), significantly potentiates the effect of submaximal concentrations of nocodazole (50-200 nM) on transendothelial electrical resistance (TER) suggesting the involvement of PP2A activity in the MT-mediated EC barrier regulation. In order to further elucidate the role of PP2A in the regulation of EC cytoskeleton and permeability, PP2A catalytic (PP2Ac), A regulatory (PP2Aa) subunits and an A subunit mutant ($PP2Aa_N$) were cloned and human pulmonary arterial EC (HPAEC) were transfected with PP2A mammalian expression constructs or infected with PP2A recombinant adenoviruses. Immunostaining of PP2Ac or PP2Aa+c overexpressing HPAEC indicated actin cytoskeleton rearrangement and co-localization of F-actin with PP2A subunits. PP2A overexpression prevented or dramatically reduced thrombin- or nocodazole-induced F-actin stress fiber formation and microtubule (MT) dissolution. It also attenuated thrombin- or nocodazole-induced decrease in transendothelial electrical resistance indicative of barrier protection. Inhibition of PP2A by okadaic acid abolished the effect of PP2A on agonist-induced changes in EC cytoskeleton indicating a critical role of PP2A activity in EC cytoskeletal maintenance. When the catalytic subunit was co-expressed with the $PP2A_N$ mutant, the actin staining exhibited no difference from the neighbouring control cells which might suggest that the mutant A subunit binds and “eliminates” the native B subunits. The catalytic subunit still may associate with the native A subunit, but this dimer form without available B subunit can not exhibit activity on substrates affecting the F-actin structure.

We demonstrated that significant amounts of PP2A, HSP27 and tau, two cytoskeletal proteins, were present in MT-enriched BPAEC fractions

indicating tight association of PP2A with MT in endothelium and with HSP27 and tau. Okadaic acid caused significant increases in tau phosphorylation confirming that tau is a substrate for PP2A in endothelium. PP2A overexpression significantly attenuated thrombin- or nocodazole-induced phosphorylation of HSP27 and tau, which potentially can be involved in agonist-induced cytoskeletal rearrangement and permeability increase. PP2A-mediated dephosphorylation of HSP27 and tau correlated with PP2A-induced preservation of HPAEC and BPAEC cytoskeleton and barrier maintenance. Collectively, our studies clearly demonstrate the crucial role of PP2A in EC barrier protection.

The thesis is based on the following publications

K. Tar, A.A. Birukova, Cs. Csortos, É.Bakó, J.G.N. Garcia, A.D. Verin: Phosphatase 2A is involved in endothelial cell microtubule remodelling and barrier regulation *J Cell Biochem. 2004 Jun 1;92(3):534-46. [IP:2,664]*

A. M. Teckchandani, A.A. Birukova, K.Tar, A.D. Verin, A. Y. Tsygankov: The multidomain protooncogenic protein c-Cbl binds to tubulin and stabilizes microtubules (*in press) Experimental Cell Research 2005 [IP:3,949]*

K. Tar, Cs. Csortos, I. Czikora, Shwu-Fan Ma, R. Wadgaonkar, P. Gergely, J.G.N. Garcia, A.D. Verin:

The role of protein phosphatase 2A in the regulation of endothelial cell cytoskeleton structure *(submitted)*

Presentations and posters

Presentations

Tar K.: A PP2A szerepe endothel sejtek citoszkeleton szerkezetének szabályozásában PhD konferencia, Debrecen, 2005

Tar K.: Protein foszfatáz 2A (PP2A) endotheliumban Magyar Tüdőgyógyász Társaság 53. Nagygyűlése 2004 jún 3-6, Debrecen

Tar, K.: Role of phosphatase 2A activity in microtubule-mediated endothelial cell cytoskeleton rearrangement. FASEB Experimental biology 2004 április Washington DC, „Graduate Student Highlights in Respiration Physiology" FASEB Journal 18 (4): A716-717 March 23, 2004 [IP:7,252]

Tar K.: A protein foszfatáz 2A (PP2A) szerepe endotheli sejtek citoszkeletonjának újrarendeződésében PhD konferencia, Debrecen, 2004

Tar K.: A protein foszfatáz 2A (PP2A) szerepe endothelium citoszkeleton szabályozásában, Debreceni Akadémiai Bizottság, Debrecen 2003. február

Tar K.: A protein foszfatáz 2A (PP2A) szerepe endothelium citoszkeleton szabályozásában PhD konferencia, Debrecen, 2003

Posters

Czikora I., Tar K., A.D. Verin, Gergely P, Csortos Cs.: Protein foszfatáz 2A alegységek overexpressziója endothel sejtekben, VI. Magyar Genetikai Kongresszus, XIII. Sejt- és Fejlõdésbiológiai Napok 2005. április10-12. Eger

Tar K., Czikora I, A.D. Verin, Gergely P., Csortos Cs.: Protein foszfatáz 2A szerepe endothel sejtek citoszkeletonjának újrarendeződésében A Magyar Biokémiai Egyesület Molekuláris Biológiai Szakosztály Munkaértekezlete Sopron, 2004. május 14-17.

K. Tar, C. Csortos, A.A.Birukova, J.G.N.Garcia and A.D. Verin: Role of phosphatase 2A activity in microtubule-mediated endothelial cell cytoskeleton rearrangement. FASEB Experimental biology 2004, Washington DC, FASEB Journal 18 (4): A716-717 March 23, 2004 [IP:7,252]

K. Tar, Cs. Csortos, A.A. Birukova and A.D. Verin: The role of protein phosphatase 2A (PP2A) in endothelial cell (EC) cytoskeleton. ATS Seattle 2003, Am. J. Respir. Crit. Care Medicine, 2003, 167,7, p.A119 [IP:6,567] 2002

Tar K., A. Verin, Gergely P., Csortos Cs: A protein foszfatáz 2A szerepe endothel sejtek citoszkeleton szerkezetének szabályozásában. 2003. okt. 10-12. Hőgyész A Magyar Biokémia Egyesület II. Jelátviteli Konferencia

Tar K. A.D. Verin, Gergely P, Csortos Cs.: Protein foszfatáz 2A szerepe endothel sejtek citoszkeleton szerkezetének szabályozásában 2003. május 12-15. Tihany, A Magyar Biokémiai Egyesület Mol. Biol. Szakosztálya 8. Munkaértekezlete

A.A. Birukova, K. Tar, Cs. Csortos, E. Bako, J.G.N. Garcia, and A.D. Verin: Role of phosphatase 2A (PP2A) in endothelial cell (EC) microtubule remodeling and barrier regulation. USA, 2002 [IP:2,527]

Tar K., Bakó É., Baranyi N., A. Verin, J.G.N. Garcia, Gergely P., Csortos Cs.: Protein foszfatáz 2A (PP2A) jellemzése endotheliumban Magyar Biokémiai Egyesület Jelátviteli Konferencia, Eger, 2001. október 11-13.

K. Tar, É. Bakó, N. Baranyi, A. Verin, J.G.N. Garcia, P. Gergely, Cs. Csortos: Characterization of protein phopshatase 2A in endothelium Protein Phosphorylation and Protein Phosphatases Marburg, Germany, 2001

Other presentation(s) and posters

T. Docsa, K. Tar, B. Toth, L. Somsak, P. Gergely: Regulation of hepatic glycogen metabolism by glycopyranosylidene-spyro-thiohydantoin. Conference of the Hungarian Physiological Society (Budapest, Hungary) 2000

T. Docsa, K. Tar, B. Toth, L. Somsak, P. Gergely: Regulation of hepatic glycogen metabolism by glycopyranosylidene-spyro-thiohydantoin in isolated hepatic cells. Conference of the Hungarian Biochemical Society (Sopron, Hungary) 2000

Tar K: Glikogén foszforiláz szabályozása izolált hepatocytában. TDK konferencia Debrecen, 2000