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

The role of calcineurin in the regulation of myosin phosphatase and endothelial barrier function

by Bernadett Kolozsvári

Supervisior: Éva Bakó, Ph.D

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2012

The role of calcineurin in the regulation of myosin phosphatase and endothelial barrier function

by Bernadett Kolozsvári
Molecular biology (M.Sc. degree)

Supervisor: Éva Bakó, Ph.D.

Doctoral School of Molecular Medicine
University of Debrecen

Head of the Examination Committee: Prof. Dr. László Csernoch, doctor of HAS
Members of the Examination Committee: Dr. Szabolcs Sipeki, Ph.D
Dr. István Szatmári, Ph. D

The Examination takes place at the Department of Physiology, Medical and Health Science Center, University of Debrecen, 2012.11.13. 11:00

Head of the Defense Committee: Prof. Dr. László Csernoch, doctor of HAS
Reviewers: Dr. László Cervenák, Ph.D
Dr. Andrea Dóczy-Bodnár, Ph.D
Members of the Defense Committee: Prof. Dr. László Csernoch, doctor of HAS
Dr. Szabolcs Sipeki, Ph.D
Dr. István Szatmári, Ph. D
Dr. László Cervenák, Ph.D
Dr. Andrea Dóczy-Bodnár, Ph.D

The Ph.D defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen, 2012.11.13. 13:00

INTRODUCTION

An important function of vascular endothelial cells (EC) is to maintain a selective barrier, thereby controlling the exchange of molecules across the wall of blood vessels. Physiological and/or pathological stimuli (e.g. inflammation, allergy, physical injury) may change the integrity of endothelial cells leading to the formation of gaps between the cells, thus compromising barrier function and increasing vessel permeability. Treatment of EC with thrombin promotes actin polymerization, and causes decrease in cortical actin filaments, while increases stress fibre formation, resulting in cell contraction.

The cytoskeletal and intercellular junction proteins of EC play an important role in the regulation of barrier integrity, and their physiological functions are often mediated by phosphorylation on Ser/Thr/Tyr residues. These findings directed attention to uncover the types of protein kinases and phosphatases implicated in the phosphorylation of key endothelial proteins involved in the mediation of endothelial permeability.

Actomyosin based contractility of EC is involved in the regulation of cell shape and stress fiber formation, which are major factors in the development of intercellular gaps during decreased barrier integrity. The contraction of EC is elicited by increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and in the phosphorylation of the 20 kDa regulatory light chain (MLC20) of nonmuscle myosin II. The latter is balanced by the actual activity ratio of MLC20 kinase (MLCK), a Ca^{2+}/calmodulin (CaM)-dependent enzyme and myosin phosphatase (MP).

Calcineurin (CN, also known as PP2B) is a Ca^{2+}/CaM-dependent Ser/Thr specific protein phosphatase. Calcineurin has been implicated in the mediation of endothelial permeability, however its cellular targets are not unambiguously identified yet. CN was shown to associate with the cytoskeletal (myosin-rich) fraction of EC and was phosphorylated and activated upon thrombin stimulation resulting in dephosphorylation of EC proteins. Pharmacological inhibition of CN by cyclosporin A (CsA) or FK506 prolonged the contractile effect and maintained gap formation between ECs. The goals of the present study were to dissect the molecular mechanism by which CN may affect the contractile machinery with special interest in the mediation of MLC20 phosphorylation by the regulation of MP.

Structure, function and regulation of myosin phosphatase

Myosin phosphatase (MP) consists of the δ isoform of protein phosphatase-1 (PP1) catalytic subunit (PP1cδ), PP1cδ-associated 130-133 kDa regulatory protein termed myosin phosphatase target subunit 1 (MYPT1), and a 20 kDa (M20) protein. Activity and substrate

specificity of the MP holoenzyme is controlled by the MYPT1 targeting subunit. Phosphorylation of Thr696 and Thr853 residues in MYPT1 results in the inhibition of phosphatase activity of PP1c. MYPT1 also serves as a protein-protein interaction platform, by providing biding site(s) for the substrates and regulatory proteins of MP in its N- and/or C-terminal region. The N-terminal region of MYPT1 is involved in the interaction with PP1c, in which the ^{35}KVKF^{38} binding motif has an essential role. The phosphorylated myosin and MLC20 bind to the N-terminal half of MYPT1 at the ankyrin repeat region.

The regulation of MP in the smooth muscle and endothelium are similar. Phosphorylation of Thr696 and Thr853 side chains by RhoA activated kinase (ROK) causes the inhibition of MP. On the other hand, phosphorylation of MYPT1 can also result in the activation of MP. Cyclic nucleotide-dependent kinases, such as the the cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) phosphorylate the Thr696 side chain of MYPT1. Although this phosphorylation of MYPT1 has no direct effect on MP activity, however, it prevents phosphorylation of Thr696 by ROK, thereby avoiding the inhibition of MP. Cyclin-dependent kinase(s) phosphorylate the central region of MYPT1, which increases the affinity of MP for myosin.

The phosphatases involved in dephosphorylation of MYPT1 are much less understood, especially in the endothelium. Protein phosphatase-2A (PP2A) and calcineurin (CN) were considered as potential MYPT1 phosphatases acting on MYPT1^{pThr696} and MYPT1^{pThr853} as revealed by *in vitro* assays. PP2A have been implicated in the *in vivo* dephosphorylation of MYPT1, since PP2A-specific inhibition using cell-permeable phosphatase inhibitory toxins (okadaic acid and calyculin-A) profoundly enhanced both MYPT1^{pThr696} and MYPT1^{pThr853} in HepG2 and THP-1 cells. The role of calcineurin in dephosphorylation of MYPT1 *in vivo* has not been proven yet.

The structure and regulation of calcineurin

The native form of CN is a heterodimer of two tightly bound subunits: calcineurin A (CNA), a 58–64 kDa catalytic subunit, and calcineurin B (CNB), a 19 kDa Ca^{2+}-binding regulatory subunit. The two-subunit structure is essential for CN activity and it includes Fe^{2+}/Zn^{2+} ions at the active center. The regulatory domain of CNA contains the CnB-binding helix, as well as the CaM-binding and autoinhibitory (AI) subdomains. CNA is represented by three isoforms (α, β γ), which are the products of alternative splicing of the CNA genes. The enzymatic activity of CN is repressed in the native protein, but it becomes fully active upon binding of Ca^{2+}/CaM at increased at increased $[Ca^{2+}]_i$ level. In unstimulated EC, at

presumably low $[Ca^{2+}]_i$, the CaM does not bind to the CNA, the enzyme is inactive. When the $[Ca^{2+}]_i$ increases the Ca^{2+}/CaM complex binds to the CNA and the enzyme activity increases. Ca^{2+}-binding to CNB is also an important event in the activation. Additional mechanisms for regulating CN involve redox reactions of active site metal ions (Fe^{2+}, Zn^{2+}) and phosphorylation of the enzyme. Ca^{2+}/CaM-dependent protein kinase II (CaMK II) can phosphorylate CNA inhibiting the Ca^{2+}/CaM-binding and resulting in reduction of the phosphatase activity.

Pharmacological agents such as cyclosporin A (CsA) and FK506, inhibit CN by forming complex with their respective cytoplasmic immunophillins, cyclophillin and FK506-binding proteins, respectively. CN has much narrower in vitro substrate specificity than the other two major Ser/Thr phosphatases, PP1 or PP2A.

AIMS

It is known from the literature, that calcineurin has an important role in the regulation of actin cytoskeleton structure of bovine and human pulmonary artery endothelial cells and in the regulation of barrier integrity. The three isoforms of calcineurin catalytic subunit (CNAα, β, γ) are equally expressed in endothelial cells and their activity increases upon thrombin treatment, however, exact functions of CN in the vascular endothelium and the detailed molecular mechanism of its action is still obscure.

Regulations of the phosphorylation of MLC20 and actin polymerization are widely investigated in endothelial model system since these are the major determinants in the control of endothelial barrier function. Relationship between these two processes is also evident as increased MLC20 phosphorylation results in barrier dysfunction only if enough polymerized actin (F-actin) is available for the formation of stress-fibers. However factors regulating actin polymerization and myosin phosphorylation have not been fully explored yet. Therefore, the possibility arises that CN may regulate the phosphorylation level of enzymes which are involved in the regulation of actin polymerization and myosin phosphorylation. CN was shown to affect the endothelial barrier function and the phosphorylation level of cytoskeletal proteins, however the molecular mechanism behind this events and the possible substrates of CN are still not fully uncovered.

Based on the above data, our studies aimed the investigation of the following questions:

1. Clarification of the role of calcineurin (CN) in the regulation of cytoskeleton structure of bovine pulmonary artery endothelial cells, using pharmacological inhibitors

(cyclosporine A/CsA and FK506) or by overexpression of the different calcineurin catalytic subunit isoforms (CNAα, CNAβ, CNAγ) as well as a constitutively active CNA form.

2. Examination if CN controls myosin phosphorylation via mediating the phosphorylation state of the inhibitory site (Thr696 and/or Thr853) in myosin phosphatase (MP) target subunit 1 (MYPT1).
3. Study and characterization of the interaction of calcineurin with MYPT1.
4. Investigation of the role a calcineurin-MYPT1 interaction in the regulation of the physiological functions in endothelial cells.

MATERIALS AND METHODS

Proteins

Glutathione-S-transferase (GST)-coupled full-length MYPT1 (GST-MYPT1$^{1-1004}$) and a C-terminal fragment (GST-MYPT1$^{667-1004}$) based on the sequence of the 133 kDa chicken gizzard isoform, and hexahistidine (His)-tagged N-terminal MYPT1 fragments (His-MYPT1$^{1-633}$, His-MYPT1$^{1-296}$, His-MYPT1$^{304-511}$) based on the sequence of the 130 kDa chicken gizzard isoform were expressed in *E. coli* and purified via the respective affinity chromatography procedures. PP1c, PP2Ac and inhibitor-2 were purified from rabbit skeletal muscle. Calcineurin was purified from bovine brain.

Cell cultures, treatments and transfections

Bovine pulmonary artery endothelial cells (BPAEC) from American Type Tissue Culture Collection and human pulmonary artery endothelial cells (HPAEC) from Clonetics were cultured and used. ECs were grown to 100% confluency and treated with effectors in serum-free medium. Cells of tsA201 from Health Protection Agency Culture Collection were used as suggested by the supplier. Confluent EC monolayers were grown in 100-mm culture plates and treated with different effectors in serum-free medium.

Cells of tsA201 were grown to 60-70% confluency and transfected with pEGFP, ΔCN-A/pEGFP, Flag-MYPT1, or CN-Aα/pEGFP plasmids using jetPEI transfection reagent according to the manufacturer's instructions. BPAECs were grown to 60-70% confluency and transfected with pEGFP, CN-Aα/pEGFP, CN-Aβ/pEGFP, CN-Aγ/pEGFP, or ΔCN-A/pEGFP plasmids using Fugene HD transfection reagent according to the manufacturer's instructions.

Western blot

Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with inert proteins, membranes were incubated with primary antibodies, followed by HRP-conjugated secondary antibodies, and the immunoreactions were detected by enhanced chemiluminescence (ECL).

Pull-down assays

Cells of tsA201 were grown on 100-mm culture dishes to 60-70% confluency and transfected with Flag-peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys)-coupled MYPT1 (Flag-MYPT1) or co-transfected with Flag-MYPT1 plus CN-Aα/pEGFP plasmids using jetPEI transfection reagent. Flag-MYPT1 as well as its associated proteins was isolated from cell lysates on anti-Flag resin according to the manufacturer's recommendations. Resin-bound proteins were solubilized by boiling in SDS sample buffer and were subjected to Western blotting using anti-MYPT1$^{1-296}$, anti-CN-Aα, anti-PP1cδ and anti-GFP antibodies.

Pull-down assays from BPAEC and HPAEC lysates were carried out on anti-Flag resin coupled with Flag-MYPT1. Flag-MYPT1 was isolated from tsA201 cell lysates and the associated proteins were removed by washing with 2 M LiBr in TBS. This Flag-MYPT1 resin, freed of associating proteins was incubated with the BPAEC or HPAEC lysates overnight at 4 ºC. The precipitated proteins were identified by Western blotting using anti-MYPT1$^{1-296}$, anti-CNAα and anti-PP1cδ antibodies.

GST-MYPT1 pull-down from BPAEC was performed by incubation of the lysates with Glutathione-Sepharose resin coupled with GST or GST-MYPT1 in binding buffer. Uncoupled proteins were removed by washing the resin twice with binding buffer. BPAEC lysates were precleared with uncoupled Glutathione-Sepharose resin and then incubated with of GST or GST-MYPT1 protein-coupled Glutathione-Sepharose resin. Bound proteins were solubilized by boiling in SDS sample buffer and were subjected to Western blot using anti-CNAα antibody.

Protein phosphatase assay

Dephosphorylation of ^{32}P-GST-MYPT1 phosphorylated by ROCK was carried out by purified CN, PP1c or PP2Ac. The released $^{32}P_i$ was determined in a scintillation counter from the supernatant after precipitation of proteins followed by centrifugation.

Surface plasmon resonance (SPR)

Interactions of MYPT1with CN or PP1c were analyzed by surface plasmon resonance (SPR)-based binding experiments using the Biacore 3000 instrument. Proteins were immobilized on sensor chip CM5 through anti-GST antibody or via primary amine groups of the proteins. The interacting molecules (CN or PP1c) were injected over the surface at different concentrations. Binding to the immobilized proteins was monitored as a sensogram where response unit (RU) values were plotted against time. The sensograms were analyzed using the BIAevalution 3.1 software.

Measurement of transendothelial electrical resistance

Transendothelial electrical resistance (TER) was measured dynamically across a confluent monolayer of BPAECs using an electrical cell-substrate impedance sensing system (ECIS, Applied Biophysics). Decrease in monolayer resistance to electrical current flow, which correlated with paracellular gap formation, was measured. BPAECs were grown to 100% confluency on sterile golden electrode arrays. Cells were then treated in serum-free medium and basal TER was measured. The values were normalized to the initial baseline resistance of each monolayer.

Immunofluorescence and confocal microscopy

After treatment with different effectors BPAECs and HPAECs were plated on coverlips. Cells were fixed with paraformaldehyde (4%) and permeabilized with 0.05% Triton X-100 dissolved in PBS. After blocking in 1% BSA/PBS, coverlips were incubated with primary antibodies diluted in 0.1% BSA/PBS, and then with Texas Red-phalloidin or Alexa 488- or Alexa 543-conjugated secondary antibodies. Finally, coverlips were covered with mounting medium using Prolong Gold Antifade Kit. Cell cultures prepared for immunofluorescence were imaged on a Nikon Eclipse TE300 fluorescens microscope or on an Olympus Fluoview 1000 confocal microscope equipped with four optical channels. Fluorescence from Alexa 488 or GFP was excited by the 488 nm line of an Ar ion laser and Alexa 546 was excited by a HeNe laser. Co-localization images were collected using sequential illumination by the two lasers to minimize spectral crosstalk between the dyes. Protein colocalization was evaluated by calculating the Pearson's correlation coefficient (C values) between the pixel intensities of the green and red channels corresponding to CN-Aα and MYPT1 signals. Prior to correlation analysis, images were filtered by a 3x3 averaging

filter to reduce noise. Cytoplasmic regions of cells were selected by ROIs excluding the nucleus.

Flow cytometry

For quantification of the phosphorylation level of $MLC20^{Ser19}$ and the possible cytotoxic effect of the transfections or treatments, and the efficiency of the transfections, were assessed by flow cytometric analysis. Cells were analyzed using a FACSCalibur instrument (Becton-Dickinson).

Ca^{2+} imaging

BPAECs were plated on sterile glass coverslips for 24 h and were grown to confluency. Cells were loaded with 5 μM Fura-2 fluorescent Ca^{2+}-sensitive dye in DPBS. Measurements were carried out in DPBS (with Ca^{2+} or with 10 mM EGTA) at room temperature. Cells were placed into a Ca^{2+}-imaging system and within a field were illuminated alternately at 340 and 380 nm and emitted light at >510 nm was measured. Data were analyzed with the InCyt 4.5 software.

RESULTS AND DISCUSSION

Effect of CN inhibitors on thrombin-induced stress fibers formation

We investigated the role of CN in thrombin-induced cytoskeleton changes in endothelial cells (BPAECs) using pharmacological inhibitors (CsA and FK506) of CN. Thrombin treatment caused drastic changes of the BPAECs actin cytoskeleton structure: it increased actin polymerization and induced stress fiber formation leading to appearance of gaps between the cells. These events were reversed when thrombin was washed out, whereas they were sustained in the presence of the CN inhibitors (CsA and FK506). These data demonstrated that CN contributed to the recovery of EC from thrombin-induced barrier dysfunction, whereas pharmacological inhibition of CN by CsA or FK506 prolonged the contractile effect and maintained gap formation between ECs.

Effect of overexpression of CNA isoforms on thrombin-induced stress fiber formation

To further clarify the role of the isoforms of CNA in the regulation of EC cytoskeleton structure, we overexpressed in BPAEC the three major isoforms of CNA (α, β, and γ) or a constitutive active truncated form (ΔCNA) which lacked the CaM-binding and autoinhibitory (AI) domains. The overexpressed pEGFP and CNA subunit isoforms had no effect on cytoskeleton structure of BPAEC either in untreated or thrombin-stimulated cells. On the other hand, transfection of BPAEC by the constitutive active, truncated form (ΔCNA-pEGFP) attenuated stress fiber formation in the presence of thrombin. These results clearly indicate that CN is involved in the regulation of EC barrier function. However, the molecular mechanism of its action is still obscure.

Effect of CN inhibitors on the phosphorylation level of Thr696 and Thr853 side chains of MYPT1 in ECs

CsA and FK506 treatments of BPEACs or HPAECs increased significantly the phosphorylation level of MYPT1 at Thr696 and Thr853 side chains which are inhibitory on the activity of myosin phosphatase. H1152, a specific ROK inhibitor, inhibited the CsA induced increase in $MYPT1^{pThr696}$ and $MYPT1^{pThr853}$ suggesting the role of ROK in these phosphorylation processes.

In *in vitro* phosphatase assays purified CN dephosphorylated recombinant GST-MYPT1 substrate (^{32}P-labeled at Thr696 and Thr853 via phosphorylation by ROK) in a dose and Ca^{2+}/CaM dependent manner. The kinetics of dephosphorylation was similar to that of

observed with PP1c and PP2Ac. Overexpression of a pEGFP-coupled constitutively active CNA form resulted in a decrease in the level of endogenous MYPT1^{pThr696} in tsA201 cells also suggesting the role of CN in MYPT1 dephosphorylation. The above data imply that CN dephosphorylates MYPT1 directly, and in a Ca^{2+}/CaM manner.

Effects of CsA on $[Ca^{2+}]_i$ and on the phosphorylation of cofilin^{Ser3}

We examined the effect of CsA on $[Ca^{2+}]_i$ of BPAEC and found that CsA induced a transient rise in $[Ca^{2+}]_i$ which was partially maintained during the incubation period with CsA. This CsA-induced $[Ca^{2+}]_i$ transient was dependent upon the presence of extracellular Ca^{2+} implicating plasma membrane Ca^{2+}-channel(s) in this process. The CsA-induced $[Ca^{2+}]_i$ transient is a prerequisite of the increase in MYPT1^{pThr696} as it is not observed in the absence of extracellular Ca^{2+} (in the presence of EGTA). The rise in $[Ca^{2+}]_i$ may also increase MYPT1^{pThr696} by the activation of ROK, since it has been shown that increasing $[Ca^{2+}]_i$ is coupled with enhancement of active RhoA (RhoA-GTP).

Previous studies have shown that an increase in $[Ca^{2+}]_i$ coupled with CN activation results in dephosphorylation and activation of Slingshot, a major cofilin^{pSer3} phosphatase, thereby lowering cofilin^{pSer3}. Elevated $[Ca^{2+}]_i$ may also increase ROK activity, which activates LIM-kinase (a cofilin kinase), thus it contributes to enhancing cofilin^{pSer3}. This elevation in cofilin^{pSer3} is further favored by CsA induced CN inhibition preventing activation of Slingshot, thus suppressing dephosphorylation. Cofilin^{pSer3} has suppressed actin-depolarizing activity; therefore a rise in cofilin^{pSer3} may contribute to increased F-actin content and actin-filament reorganization in EC.

***In vivo* and *in vitro* interaction of CN and MYPT1**

To analyze if MYPT1 and CN interact with each other in a physiologically relevant way pull-down experiments with GST-MYPT1 and Flag-MYPT1 in BPAEC and HPAEC lysates containing endogenous or overexpressed CN, were carried out. Nonphosphorylated GST-MYPT1 pulled down the CNA subunit from endothelial cell lysate indicating that interaction may develop between these proteins without the necessity of phosphorylation of MYPT1. Confocal microscopy localized both CNAα and MYPT1 predominantly in the cytoplasm and to a lesser extent in the nucleus in BPAEC and HPAEC. The appearance of merged images suggested colocalization of these proteins in both cell types, which was confirmed by the determination of the Pearson's correlation coefficients and it was apparently unaffected upon thrombin treatments of the cells.

To clarify the molecular background for the formation of the CN-MYPT1 complex, binding of CN to full-length and truncated mutants of MYPT1 was determined in SPR-based experiments using purified proteins. Our findings confirm that CN forms a stable complex with dephosphorylated MYPT1 (K_a~10^7). SPR-binding studies establish the essential role of the N-terminal region of MYPT1 in the interaction with CN. Accordingly, there is a CN-substrate-docking PxIxIT-like motif in the N-terminal region of MYPT1 (^{300}PLIEST^{305}), which may play an important role in the interaction with CN. Possible competition between PP1c and CN was assessed since PP1c was also shown to bind to the N-terminal MYPT1 region. However, competitive binding sites for PP1c and CN were excluded as the two proteins bind independently to MYPT1 surface in SPR binding studies suggesting that CN presumably interacts with the MP holoenzyme (PP1c-MYPT1) with the same affinity as does with MYPT1.

The role of interaction of calcineurin and MYPT1 in the regulation of myosin phosphatase and endothelial barrier function

The level of MYPT1^{pThr696} is a key factor in the activity status of MP and it controls MLC20 phosphorylation. CN was shown to associate with the cytoskeletal (myosin-rich) fraction of EC and was phosphorylated and activated upon thrombin stimulation resulting in dephosphorylation of EC proteins. In contrast, in the presence of CN inhibitors EC protein dephosphorylation including MLC20^{pSer19} was attenuated. Our results support a mechanism in which CN counterbalances the phosphorylation of the inhibitory site(s) in MYPT1 keeping MP in an active state, thereby decreasing MLC20^{pSer19}. Thrombin triggers a rise in $[Ca^{2+}]_i$ which activates both MLCKs and CN resulting in transient MLC20 phosphorylation and MP inhibition. Our present data imply that the transient MP inhibition is coupled with a transient increase in MYPT1^{pThr696}. The role of CN in mediating MYPT1^{pThr696} dephosphorylation during thrombin stimulation is confirmed by the findings that pretreatment of EC with CsA results in partially sustained phosphorylation of both MYPT1^{pThr696} and MLC20^{pSer19}. However, CN is not the sole phosphatase for MYPT1 dephosphorylation as indicated by the only partially sustained phosphorylation upon CN inhibition. The role of PP1c and PP2Ac in MYPT1^{pThr696} dephosphorylation is also shown by in vitro phosphatase assays, but in cells they may act in holoenzyme forms, which are still not identified.

Effect of inhibition of calcineurin on EC barrier function

It has been shown that CN inhibitors (CsA, FK506, deltamethrin) influence EC permeability. FK506 alone did not alter TER, but it prevented the reversal of thrombin-induced decrease in resistance, while PP1 and PP2A inhibitors were without effect. Our results suggest that CN inhibition by CsA decreases TER in BPAEC to a significant extent without a profound change in MLC20 phosphorylation. We hypothesize that this change in TER by CsA is due to an increase in cofilin^{pSer3} resulting in enhanced actin polymerization with subsequent alterations in EC cell shape and permeability. CsA treatment also slows down the recovery of decreased TER induced by thrombin and this effect appears to parallel the sustained level of MYPT1^{pThr696} and MLC20^{pSer19}. Our data imply that CN inhibition by CsA exerts both MLC20 phosphorylation-dependent and independent effects.

CONCLUSIONS

- We have provided evidence for the involvement of calcineurin activity in decreasing thrombin-induced stress fiber formation in endothelial cells, while calcineurin inhibitors exerted opposite effects. These results suggest that calcineurin mediates cell contractility via inducing changes in the cytoskeletal structures.
- We have identified calcineurin as a phosphatase which dephosphorylate MYPT1 at the inhibitory phosphorylation sites, Thr696 and Thr853 in a Ca^{2+}/CaM-dependent manner.
- We detected formation of stable complexes between dephosphorylated MYPT1 (and its fragments) and CN. These data suggest that beside coupled in an enzyme-substrate adduct MP (even via dephosphorylated MYPT1) forms a stable interaction with CN which may have physiological significance in other signaling complexes, too.
- In conclusion, our present data suggest that calcineurin is involved in the recovery of ECs from thrombin-induced dysfunction, presumably via activation of MP by dephosphorylation of MYPT1 thereby decreasing the phosphorylation level of myosin.

Experimental work was performed by the support of the TÁMOP-4.2.2.A-11/1/KONV-2012-0025 project. The doctoral training program was supported by the TÁMOP-4.2.2/B-10/1-2010-0024 project. The projects are co-financed by the European Union and the European Social Fund.

Register Number: DEENKÉTK/275/2012.
Item Number:

Subject: Ph.D. List of Publications

Candidate: Bernadett Kolozsvári

Neptun ID: DADHZV

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Kolozsvári, B.**, Bakó, É., Bécsi, B., Kiss, A., Czikora, Á., Tóth, A., Vámosi, G., Gergely, P., Erdődi, F.: Calcineurin regulates endothelial barrier function by interaction with and dephosphorylation of myosin phosphatase.
   Cardiovasc. Res. Epub ahead of print (2012)
   DOI: http://dx.doi.org/10.1093/cvr/cvs255
   IF:6.064 (2011)

2. **Kolozsvári, B.**, Szíjgyártó, Z., Bai, P., Gergely, P., Verin, A., Garcia, J.G.N., Bakó, É.: Role of calcineurin in thrombin-mediated endothelial cell contraction.
   Cytometry A. 75 (5), 405-411, 2009.
   DOI: http://dx.doi.org/10.1002/cyto.a.20707
   IF:3.032

List of other publications

3. Kiss, A., Bécsi, B., **Kolozsvári, B.**, Komáromi, I., Kövér, K.E., Erdődi, F.: Epigallocatechin-3-gallate and penta-O-galloyl-beta-d-glucose inhibit protein phosphatase-1.
   FEBS J. Epub ahead of print (2012)
   DOI: http://dx.doi.org/10.1111/j.1742-4658.2012.08498.x
   IF:3.79 (2011)

4. Matta, C., Juhász, T., Szíjgyártó, Z., **Kolozsvári, B.**, Somogyi, C., Nagy, G., Gergely, P., Zákány, R.: PKCdelta is a positive regulator of chondrogenesis in chicken high density micromass cell cultures.
Biochimie. 93 (2), 149-159, 2011.
DOI: http://dx.doi.org/10.1016/j.biochi.2010.09.005
IF:3.022

5. Juhász, T., Matta, C., Mészár, Z., Nagy, G., Szíjgyártó, Z., Molnár, Z., **Kolozsvári, B.**, Bakó, É., Zákány, R.: Optimalized transient transfection of chondrogenic primary cell cultures.
Cent. Eur. J. Biol. 5 (5), 572-584, 2010.
DOI: http://dx.doi.org/10.2478/s11535-010-0053-x
IF:0.685

Total IF: 16.593

Total IF (publications related to the dissertation): 9.096

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.

11 September, 2012