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Phosphatase 2A Is Involved in Endothelial Cell Microtubule Remodeling and Barrier Regulation

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Abstract We have recently shown that microtubule (MT) inhibitor, nocodazole (2–5 μM) significantly increases endothelial cells (EC) actomyosin contraction and permeability indicating the importance of MT in maintaining the EC barrier (Verin et al. [2001]: Cell Mol Physiol 281:L565–L574). Okadaic acid (OA, 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. Immunofluorescent staining of EC revealed that in control cells PP2A distributes in a pattern similar to MT. Consistent with these results, we demonstrated that significant amounts of PP2A were present in MT-enriched EC fractions indicating tight association of PP2A with MT in endothelium. Treatment of EC with OA leads to disappearance of MT-like PP2A staining suggesting dissociation of PP2A from the MT network. Next, we examined the effect of PP2A inhibition on phosphorylation status of MT-associated protein tau, which in its unphosphorylated form promotes MT assembly. OA caused significant increases in tau phosphorylation confirming that tau is a substrate for PP2A in endothelium. Immunofluorescent experiments demonstrated that the OA-induced increases in tau phosphorylation strongly correlated with translocation of phospho-tau to cell periphery and disassembly of peripheral MT. These results suggest the involvement of PP2A-mediated tau dephosphorylation in alteration of EC MT structure and highlight the potential importance of PP2A in the regulation of EC the MT cytoskeleton and barrier function. J. Cell. Biochem. 92: 534–546, 2004.

Key words: endothelium; phosphatases; permeability; microtubules; okadaic acid; nocodazole; endothelial cytoskeleton

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 syndrome and is the result of intercellular gap formation evoked by bioactive agents [Garcia et al., 1986, 1995]. 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 [for review see Dudek and Garcia, 2001]. Acto-myosin interaction and cell contractility depends upon the phosphorylation state of myosin light chains (MLC) [Garcia et al., 1986, 1995]. Increased levels of MLC phosphorylation are followed by actin redistribution, F-actin filament formation, and EC barrier dysfunction. We have previously shown that protein phosphatase 1 (PP1) associates with myosin filaments and is directly involved in EC

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contractility and barrier regulation through the dephosphorylation of MLC [Verin et al., 1995, 2000]. In addition, we have cloned and characterized several isoforms of the endothelial MLC kinase (MLCK) which is the major known enzyme affecting level of MLC phosphorylation [Garcia et al., 1997; Verin et al., 1998a,b].

Information is limited about the linkage between the microfilament network and the other two major components of the cytoskeleton, the microtubules (MTs) and the intermediate filaments. It has been shown that disruption of MTs by either colcemid, nocodazole, or vinblastine caused a rapid and substantial strengthening of fibroblast contractility [Wang et al., 1995] which was abolished by pretreatment with paclitaxel (taxol), which promotes MT assembly [Wang et al., 1995]. Subsequent studies showed that nocodazole-induced isometric contraction in fibroblasts correlated well with the level of nocodazole-induced MLC phosphorylation [Danowski, 1989]. Based on these data, it was proposed that MLC phosphorylation-driven contraction may be produced by MT inhibitors and receptor-mediated agonists [Kolodney and Elson, 1995]. Indeed, we found increased EC stress fiber content and MLC phosphorylation after MT disruption [Verin et al., 2001]. However, the exact mechanisms by which MT disruption affects EC microfilament cytoskeleton is unclear.

Several recent studies have demonstrated the significance of not only PP1, but also PP2A in maintaining EC cytoskeletal organization and barrier function [Diwan et al., 1997; Gabel et al., 1999; Knapp et al., 1999], although PP2A is not involved directly in in vivo MLC dephosphorylation. PP2A comprises a major family of Ser/Thr protein phosphatases, containing a well-conserved catalytic subunit, the activity of which is highly regulated. PP2A is a heterotrimer enzyme, composed of the structural A (65 kDa) and catalytic C (36 kDa) subunits forming the constitutive core of the enzyme, which associates with one of the large number of regulatory B subunits [Csortos et al., 1996; Janssens et al., 2001]. Several studies support the involvement of PP2A in the dephosphorylation of several MT-associated proteins (MAPs), like tau, which cause cytoskeletal remodeling suggesting their participation in the regulation of EC contraction/relaxation [Sontag et al., 1995, 1999; Litersky et al., 1996; Gong et al., 2000a; Hiraga and Tamura, 2000; Kobayashi et al., 2001]. Other studies show a notable link between PP2A activity, cytoskeleton, and heat shock proteins (HSPs) which have a protective effect during myocardial ischemia, as specific HSPs preserve the MTs during simulated cardiac ischemia [Bluhm et al., 1998].

The exact role of PP2A in the regulation of EC cytoskeleton is virtually unexplored. We hypothesized that PP2A, via changes in MAPs phosphorylation, may regulate interaction of MAPs with MT, and actin cytoskeletal components, and may directly participate in agonist-induced cytoskeletal rearrangement and barrier dysfunction.

MATERIALS AND METHODS

Reagents

Unless specified, reagents were obtained from Sigma (St. Louis, MO). Okadaic acid (OA, sodium salt) was purchased from Calbiochem (San Diego, CA). Monoclonal antibodies against β-tubulin, HSP27 and PP2A were purchased from CRP, Inc. (Covance Research Products, Denver, PA), Cell Signaling Technology (Beverly, MA), and BD Biosciences Pharmingen (San Diego, CA), respectively. Tau-specific monoclonal and phospho-tau [pS262]-specific polyclonal antibodies were from Biosource International (Camarillo, CA), PP1 and PP2B polyclonal antibodies both were purchased from Chemicon International (Temecula, CA). Texas Red-phalloidin and Alexa 488-, Alexa 594-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR).

Cell Cultures

Bovine pulmonary artery endothelial cells (BPAEC) were obtained frozen at 16 passage from American Type Tissue Culture Collection (Rockville, MD; culture line-CCL 209), and were utilized at passages 19–24 as previously described [Stasek et al., 1992]. Cells were maintained in Medium 199 (Gibco-BRL, Chagrin Falls, OH) supplemented with 20% (v/v) colostrums-free bovine serum (Irvine Scientific, SantaAna, CA), 15 μg/ml EC growth supplement (Collaborative Research, Bedford, MA), 1% antibiotic, and antimycotic solution (penicillin, 10,000 U/ml; streptomycin, 10 μg/ml; and amphotericin B 25 μg/ml; K.C. Biologicals, Lenexa, KA), and 0.1 mM non-essential amino acids (Gibco-BRL). Human pulmonary artery

endothelial cells (HPAEC) were obtained from Clonetics, BioWhittaker, Inc. (Frederick, MD) 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) with 10% FBS. Cells were used at passages 6–10. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Measurement of Transendothelial Electrical Resistance (TER)

The cellular barrier properties were monitored using the highly sensitive biophysical assay with an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) described previously [Garcia et al., 1997a; Schaphorst et al., 1997]. Cells were cultured on small gold electrodes (10^{-4} cm^2), and culture media was used as electrolyte. The total electrical resistance was measured dynamically across the 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. As cells adhere and spread out 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]. The small gold electrodes and the larger counter electrodes (1 cm^2) were connected to a phase-sensitive lock-in amplifier (5301A; EG&G Instruments, Princeton, NJ) with a built-in differential preamplifier (5316A; EG&G Instruments). A 1 V, 4,000 Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant-current source. Voltage and phase data were stored and processed with a Pentium 100 MHz computer that controlled the output of the amplifier and relay switches to different electrodes. Experiments were conducted only on wells that achieved >1,000 Ω (10 microelectrodes/well) of steady-state resistance. Resistance was expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized resistance value, as previously described [Garcia et al., 1997a].

EC Fractionation

EC fractionation was performed using two different fractionation protocols. Initial protocol allows to separate three cellular fractions, which include MT-enriched, cytoskeletal (detergent insoluble F-actin-enriched), and cytosolic fractions, which were prepared as it was previously described [Verin et al., 1998c; da Costa et al., 2000]. Briefly, EC monolayers were washed with PBS, and then the cells were incubated on ice for 30 min to promote release of polymerized tubulin into the soluble fraction. The chilled cells were scraped and resuspended in lysis buffer containing PEM (35 mM Pipes pH 7.4, 1 mM EGTA, 5 mM $MgSO_4$), 50 mM NaF, 0.4 mM Na_3VO_4, 1 mM DTT, and protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). The cell lysate was passed ten times through a 26-G needle. The cell debris was removed by centrifugation at 800*g* for 10 min at 4°C. The supernatant was further centrifuged at 92,000*g* for 30 min at 4°C. The pellet was the cytoskeletal fraction (CSK). MTs were presented in the supernatant, and were polymerized in the presence of 20 μM taxol and 1 mM GTP at 37°C for 1 h. MTs were then pelleted by centrifugation at 92,000*g* for 30 min at room temperature over a 20% sucrose gradient in PEM containing 1 mM DTT, protease inhibitor cocktail, 0.4 mM Na_3VO_4, and 20 μM taxol.

Second (more advanced, but more complex) protocol allows to separate cytosolic, tubulin, actin, and intermediate filaments (vimentin)-enriched fractions [Ding et al., 1996]. Briefly, EC monolayers were detached by an incubation in 2 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA) in phoshate buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) for 10 min at 37°C. Then cells were scraped and collected by centrifugation at 800*g* for 5 min, and resuspended in a cytoskeleton-stabilizing buffer (CSK-buffer): 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), pH 6.8, containing 250 mM sucrose, 3 mM $MgCl_2$, 150 mM KCl, 1 mM EGTA, and 1 mM phenylmethylsulphonyl fluoride (PMSF). Cytosolic fraction was isolated after 5 min incubation at room temperature in lysis buffer (0.15% Triton X-100 in CSK-buffer) by centrifugation at 14,000*g* for 10 min at room temperature. The supernatant was the cytosolic fraction (CSL). The pellet was washed three times with lysis buffer at 37°C,

and tubulin was depolymerized by chilling the washed pellet to 4°C for 20 min in lysis buffer. The sample was than centrifuged at 14,000*g* for 10 min at 4°C resulting in a tubulin-rich supernatant, the tubulin fraction (T). The actin-rich pellet was washed twice with cold lysis buffer and the actin was solubilized in solubilization buffer (0.6 M KCL, 0.2 mg/ml DNase, and 10 mM $MgCl_2$ in CSK-buffer). The actin-rich fraction (A) was separated from the intermediate filaments (vimentin, V) containing pellet fraction by centrifugation at 14,000*g* for 20 min at 4°C.

Western Immunoblotting

Protein samples were separated by SDS–PAGE [Laemmli, 1970] on 10% gels, transferred to nitrocellulose membrane (30 V for 18 h or 90 V for 2 h) as described [Towbin et al., 1992], and incubated with specific antibodies of interest. Immunoreactive proteins were detected with enhanced chemiluminescent detection system (ECL) according to the manufacturer's directions (Amersham, Little Chalfont, UK).

Immunofluorescent Staining

After specific treatments, EC grown on glass coverslips were fixed in 3.7% formaldehyde solution in PBS for 10 min at 4°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. Incubation with antibodies was performed in blocking solution for 1 h at room temperature. Alexa 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 5.0 program.

Image Analysis of Gap Formation

Images (16-bit) of the immunofluorescent staining experiments were analyzed using MetaVue 4.6 program (Universal Imaging, Downington, PA). Images were differentially segmented between gaps (black) and cells (highest grey value) based on image grayscale levels. The gap formation was expressed as a ratio of the gap area to the area of the whole image. The values were statistically processed using Sigma Plot 7.1 (SPSS Science, Chicago, IL) software.

Statistical Analysis

Results are expressed as means ± SD of the three to five independent experiments. Stimulated samples were compared with controls by unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of PP2A Inhibition on TER and Actomyosin Remodeling

We have shown that MT disassembly significantly increases EC actomyosin contraction and permeability indicating that like microfilaments, MTs also play crucial role in maintaining the EC barrier [Verin et al., 2001]. Consistent with these data, Figure 1 demonstrates that the MT inhibitor, nocodazole, significantly increases permeability (decreases TER) in a dose-dependent manner in both bovine and human EC (maximal response at 5 and 1 μM, respectively). To clarify whether PP2A, one of the major major Ser/Thr protein phosphatases, affects EC barrier function, we used OA, a specific inhibitor of PP2A activity, in combination with submaximal doses of nocodazole. BPAEC were pretreated with either vehicle (0.1% DMSO), or OA (5.0 nM) at 1 h of TER measurement, then vehicle (0.1% DMSO), or nocodazole (0.2 μM) were added at 4 h, Figure 1C demonstrate that OA alone falls to alter TER. However, OA significantly potentiates the effect of submaximal concentrations of nocodazole on TER suggesting the participation of PP2A activity in the regulation of MT-mediated EC barrier.

Consistent with these results, immunostaining of HPAEC for F-actin demonstrates that treatment of EC with OA (5 nM) did not change the actin cytoskeleton compared to the control cells, and more importantly EC gap formation was not observed (Fig. 2A,B). Nocodazole at submaximal concentration (50 nM) slightly increased stress fiber formation and evoked appearance of small paracellular gaps (Fig. 2C). In contrast, pretreatment of cells with OA significantly increased nocodazole-induced formation of paracellular gaps (Fig. 2D) confirming possible involvement of PP2A activity in EC barrier regulation. Importantly, that morphometric analysis of the immunostained images

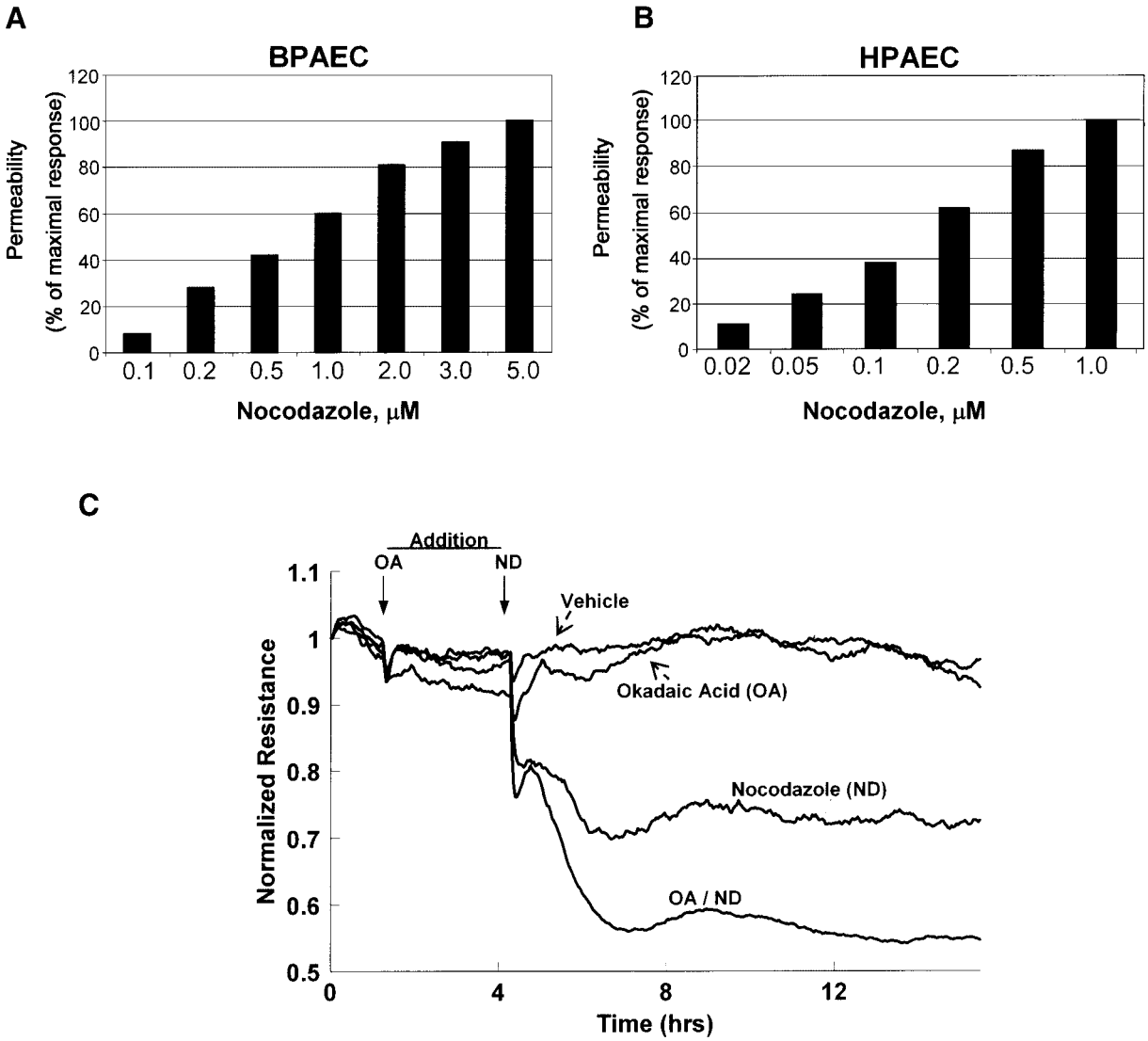
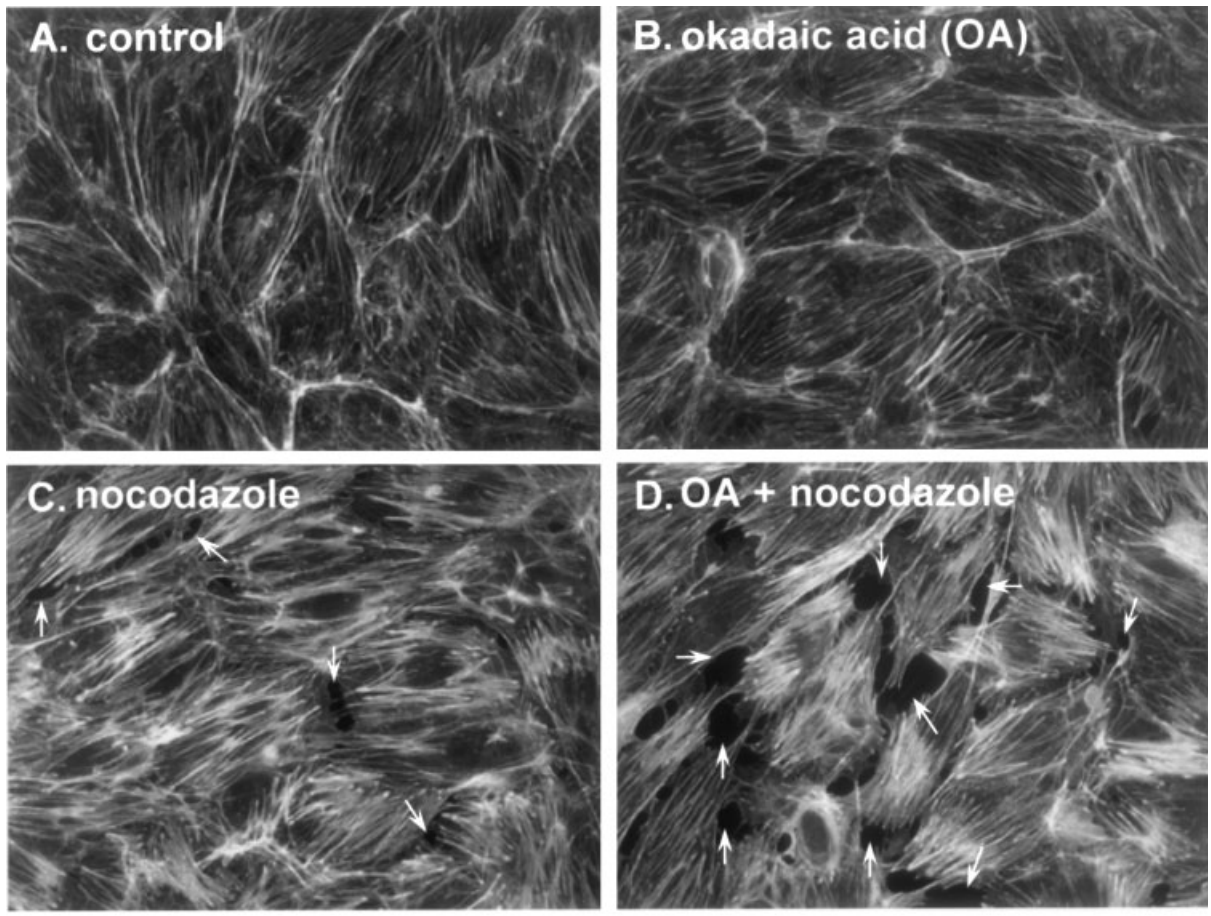


Fig. 1. Effect of protein phosphatase 2A (PP2A) inhibition and/or microtubule (MT) disruption on transendothelial electrical resistance (TER). Concentration-dependent permeability changes of bovine pulmonary artery endothelial cells (BPAEC, **panel A**) and human pulmonary artery endothelial cells (HPAEC, **panel B**) monolayers measured by electrical cell-substrate impedance sensing system (ECIS). Permeability is expressed as % of maximal response. **Panel C**: BPAEC monolayers were pretreated with either 0.1% DMSO (vehicle) or 5 nM okadaic acid (OA) in complete medium, then treated with either vehicle or 0.2 μM nocodazole (ND). TER of the monolayers was monitored for 16 h as described in "Materials and Methods." Solid arrows indicate the time of agonist addition. Results are from three representative experiments.

(Fig. 2E) showed statistically significant increases in gap area formed after combined OA/nocodazole treatment when compared to nocodazole alone.

PP2A Inhibition Alters Subcellular Localization of PP2A and MT Structure

As these results linked PP2A activity, MT and F-actin cytoskeleton remodeling, we next examined PP2A co-localization with either MTs or microfilaments in endothelium. Immunostaining detected rather similar distributions of MTs and PP2A in untreated HPAEC using β-tubulin and PP2A specific antibodies, respectively (Fig. 3A,C). Inhibition of PP2A by OA (5 nM for 1.5 h) caused dissolution of peripheral MTs, which was accompanied by changes in PP2A immunostaining from MT-like to punctate pattern (Fig. 3C,D) suggesting dissociation of PP2A from MTs after the PP2A inhibition. Thus, OA-induced subcellular redistribution of PP2A correlates well with the disappearance of peripheral MTs suggesting the involvement of PP2A activity in stabilization of MT network.



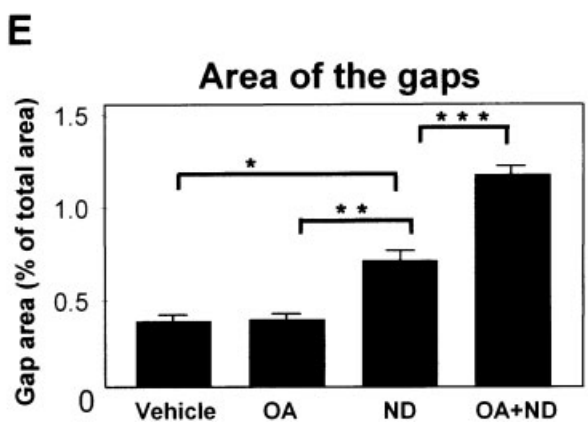


Fig. 2. PP2A inhibition potentiates endothelial cell (EC) nocodazole-induced gap formation. HPAEC monolayers were pretreated with either 0.1% DMSO (**panels A**, **C**), or 5 nM OA (**panels B**, **D**) for 1.5 h, then treated with either vehicle (panels A, B) or 50 nM nocodazole (panels C, D) for 30 min. After the indicated time of treatment the cells were fixed and F-actin was stained as described in ''Materials and Methods.'' Arrows on panels C and D show intercellular gaps. **Panel E**: The sizes of the intercellular gaps were evaluated by morphometric analysis of Texas Red-phalloidin stained HPAEC using MetaVue software. Significant difference ($P < 0.05$) was found between different groups as follows: *, compared ND treated group with vehicle; **, between ND treated group and OA treated group; ***, between ND treated group and OA + ND treated group.

Subcellular Localization of Ser/Thr Protein Phosphatases in Bovine Endothelium

Further verification of the association of PP2A with MTs was obtained by cell fractionation followed by immunoblotting. Cytosolic (CSL), MT-enriched, and cytoskeletal (CSK) F-actin-enriched fractions were separated from BPAEC as described in "Materials and Methods." Initially, we stained immunoblots

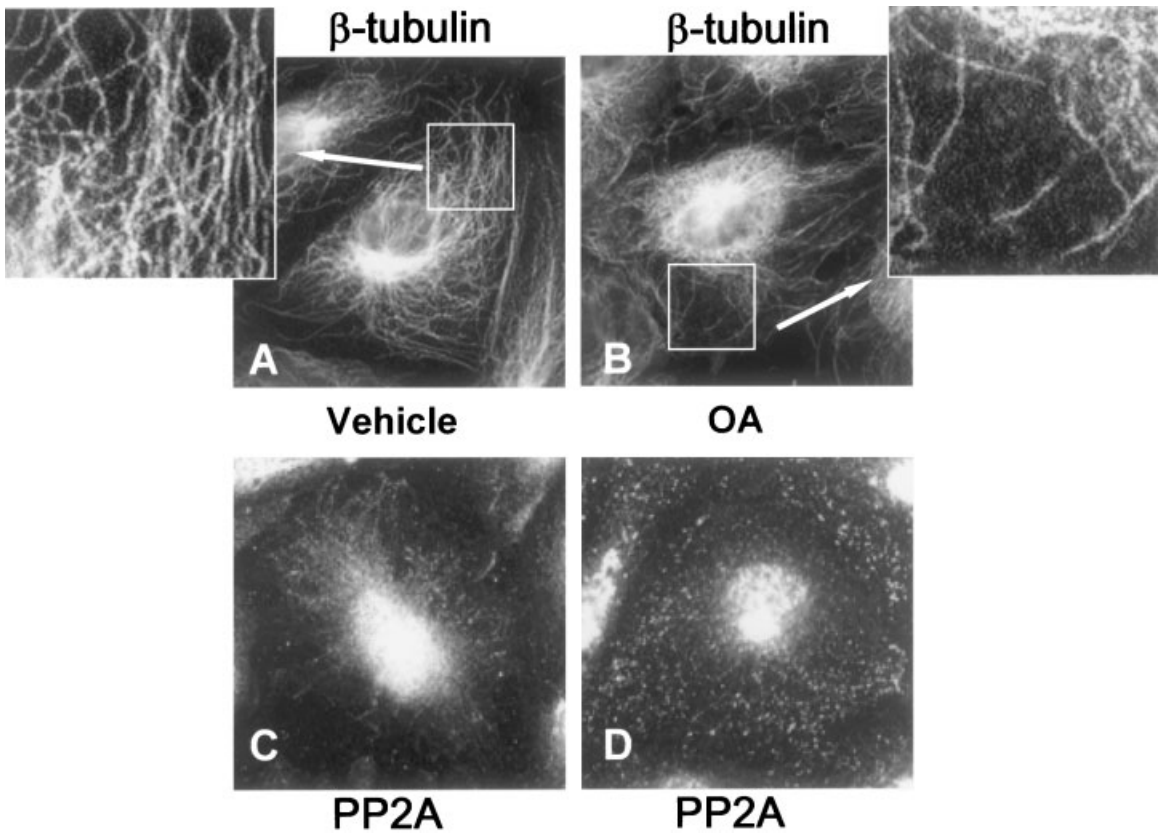


Fig. 3. PP2A inhibition alters cellular localization of PP2A and MT structure. HPAEC were treated with 0.1% DMSO (vehicle, **panels A**, **C**), or 5 nM OA (**panels B**, **D**) for 1.5 h. Cells were fixed and immunostained as described in ''Materials and Methods.'' Panels A, B: Cells were stained with β-tubulin-specific monoclonal antibody. Panels C, D: Cells were stained with PP2A-specific monoclonal antibody. Insets represent enlarged peripheral regions of EC.

with β-tubulin and tau antibodies to validate the method for preparation of MT fraction. Western blot analysis shows that MT fraction was in fact enriched in tubulin and MT-associated protein tau confirming that the isolation method is appropriate (Fig. 4A). Further immunoblotting analysis of the obtained EC fractions revealed that the majority of PP2A and its putative substrate, HSP27, were also associated with MT fraction in EC (Fig. 4, panel A) indicating tight association of these proteins with MTs in endothelium.

To further confirm the physical association of PP2A with MTs we employed another isolation method to separate actin (A)-, tubulin (T)-, vimentin (V)-enriched, and cytosolic (CSL) fractions as described in "Materials and Methods." Figure 4, panel B demonstrates again that the majority of PP2A is tightly associated with the T fraction. Staining the same fractions with antibodies specific to other Ser/Thr phoshatases revealed that PP1 is almost evenly distributed in all fractions, whereas PP2B was enriched in A and V fractions. These data indicated that only PP2A, but not other major Ser/Thr phosphatases, is specifically and tightly bound with MT suggesting the potential importance of PP2A in the regulation of EC MT cytoskeleton.

Effect of PP2A Inhibition on Tau Phosphorylation and Subcellular Localization

Putative substrate for PP2A, MAP tau in its dephophorylated form, promotes MT assembly [Drewes et al., 1998; Cassimeris and Spittle, 2001]. Therefore, next we examined the effect of PP2A inhibition on the phosphorylation status of tau in EC. We prepared total cell lysates, MT-enriched and cytosolic (CSL) fractions as described in "Materials and Methods" from control BPAEC or from BPAEC treated with either OA (specific inhibitor of PP2A at 5 nM) or calyculin A (cell-permeable inhibitor of both PP1 and PP2A, 1 nM). OA treatment resulted in a significant increase in the phosphorylation

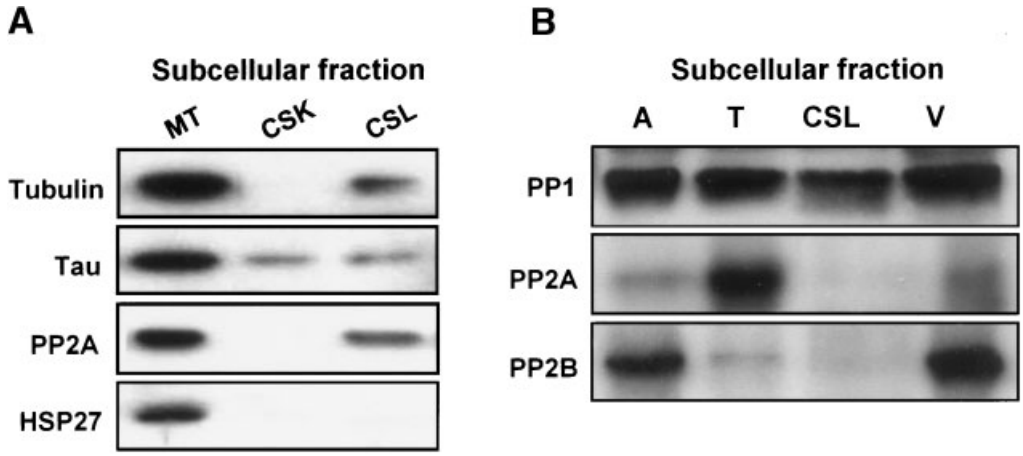


Fig. 4. Subcellular distribution of Ser/Thr-specific protein phosphatases and MAPs in endothelium. **Panel A**: Proteins from the MT, F-actin-enriched cytoskeletal (CSK), and cytosolic (CSL) BPAEC fractions prepared as described in ''Materials and Methods,'' were separated by 10% SDS–PAGE followed by Western blot using either β-tubulin-, tau-, PP2A-, or HSP27-specific antibodies. Immunoreactive protein bands were visualized by ECL technique. **Panel B**: Proteins from cytosolic (CSL), tubulin (T), actin (A), and intermediate filaments (vimentin, V) BPAEC fractions (detailed description see in ''Materials and Methods'') were separated by 10% SDS–PAGE followed by Western blot using either PP1-, PP2A-, or PP2B-specific antibodies. Immunoreactive protein bands were visualized by ECL technique. The amount of proteins loaded on the gel in each EC fraction was equivalent and represented ~25 μl of the initial cell homogenate.

level of tau, as it is shown on Figure 5 (panels A, B) suggesting that tau is a substrate of PP2A in endothelium. A specific inhibitor of both PP1 and PP2A, calyculin A did not further increase the phosphorylation level of tau indicating a limited role of PP1 in tau dephosphorylation. Interestingly, the majority of phosphorylated tau has not associated with MT fraction, a

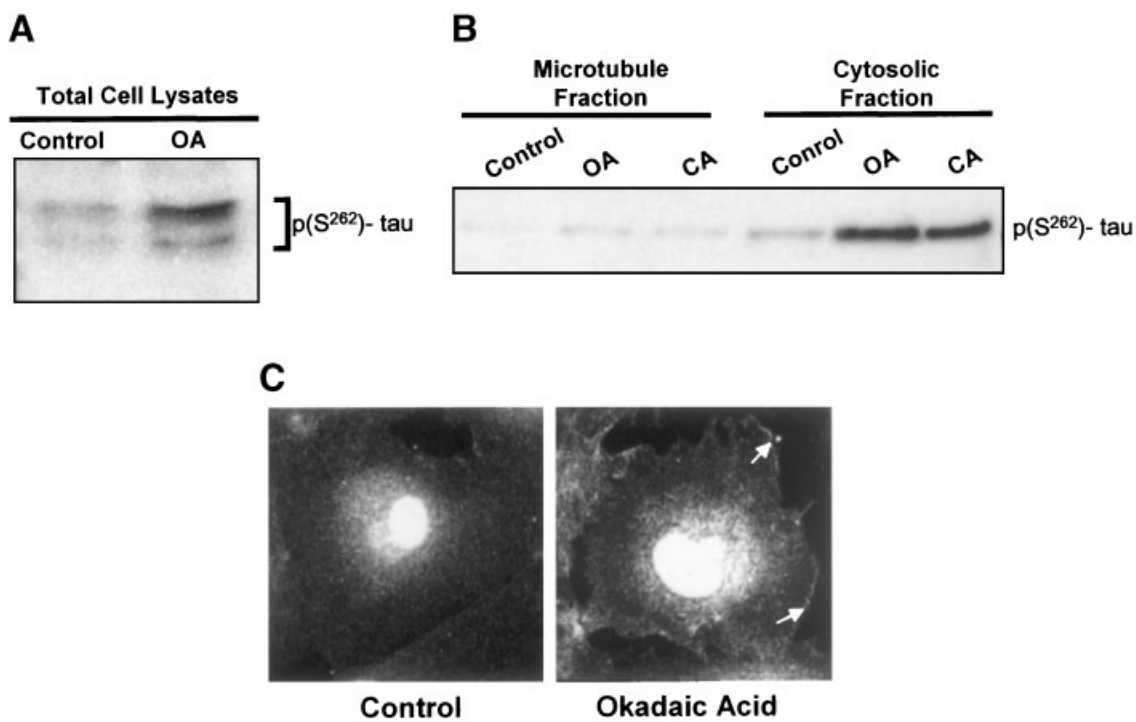


Fig. 5. Effect of PP2A inhibition on tau phosphorylation and subcellular localization. BPAEC were treated with either 0.1% DMSO (control), 5 nM OA for 1.5 h, or 1 nM calyculin A (CA) for 1 h. **Panel A**: Western blot analysis of phospho(Ser262)-tau protein content in EC total cell lysate in control or OA-treated cells. **Panel B**: Immunoblotting analysis of phospho-tau content in MT-enriched, and cytosolic fractions in control cells or after OA or calyculin (CA) treatment. **Panel C**: Immunofluorescent detection of phospho(Ser262)-tau in control and OA (5 nM)-treated HPAEC.

finding, consistent with the data of literature indicating that tau phosphorylation weakens its interaction with MTs [Drewes et al., 1998; Cassimeris and Spittle, 2001]. Immunofluorescent experiments demonstrated (Fig. 5C) that the OA-induced increases in tau phosphorylation correlated well with the translocation of phospho-tau to the cell periphery and disassembly of peripheral MTs (Fig. 3B), suggesting a role for PP2A-mediated tau dephosphorylation in EC MT assembly/disassembly.

DISCUSSION

Besides direct or indirect protein–protein interactions among the three major elements of the cytoskeleton, microfilaments, MTs, and intermediate filaments [Lee and Gotlieb, 2003], recent results suggest that direct linkage between MTs and microfilaments is important for coordinated signaling, which affect changes in EC cytoskeletal architecture [Verin et al., 2001; Niggli, 2003]. Although the molecular events linking the cytoskeletal changes initiated by MT disruption and cell contraction were not characterized in details, we have recently demonstrated a tight correlation between MT disruption, activation of the contractile machinery (MLC phosphorylation, stress fiber formation), and barrier dysfunction. The increased MLC phosphorylation after MT disruption in EC suggests activation of specific biochemical signaling pathways that lead to contraction and EC barrier failure [Verin et al., 2001].

The purpose of this work was to elucidate the role of PP2A in the regulation of EC MT cytoskeleton and permeability using pharmacological inhibitors to either disrupt MT organization (nocodazole), or to inhibit PP2A activity (OA). Nocodazole is a synthetic antitubulin agent that reversibly blocks the assembly of tubulin and depolymerizes preformed MTs [De Brabander et al., 1981]. OA 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 [Haystead et al., 1989]. OA 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) [Cohen et al., 1990]. OA up to 1 μM does not have any detectable effect on the major Ser/Thr phosphatase activities in living cells except PP2A, and therefore, can be utilized for studying PP2A function [Wera and Hemmings, 1995].

We have previously shown that OA at 2–5 nM concentration had no significant effect on bovine EC permeability and MLC phosphorylation [Verin et al., 1995] suggesting a limited role of PP2A alone in direct regulation of EC contractility and barrier function. We have also shown that OA up to 30 nM concentration inhibited only 40% of total Ser/Thr protein phosphatase activity with ^{32}P-phosphorylase a substrate, which can be attributed to PP2A activity in BPAEC homogenates [Verin et al., 1995]. On the other hand, we have shown, that MT destabilization by nocodazole (2–5 μM) significantly increased EC actomyosin contraction and permeability indicating the importance of MTs in maintaining the EC barrier [Verin et al., 2001]. Since PP2A was reported to bind and dephosphorylate several MAPs like tau, which may affect MT structure [Sontag et al., 1995, 1996, 1999; Gong et al., 2000a,b; Hiraga and Tamura, 2000; Kobayashi et al., 2001], we examined the role of PP2A in MT stability in nocodazole-induced contractile responses and permeability in EC. Our data indicated that OA (5 nM) significantly potentiated the effect of submaximal 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 MTs.

Immunofluorescent staining of EC with PP2A antibody revealed MT-like pattern in quiescent cells. Consistent with this observation, co-localization of PP2A and MTs was also shown previously in intact neuronal and non-neuronal cells based on immunofluorescent studies [Sontag et al., 1995].

MT-like pattern is completely disappeared after treatment of cells with OA. Changes in PP2A subcellular localization after PP2A inhibition coincide with disappearance of peripheral MTs. Taken together these results suggested that inhibition of PP2A phosphatase activity caused dissociation of PP2A from MTs and destabilization of MT network.

To confirm association of PP2A with MTs 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/MT-enriched fractions. The occurrence/distribution of PP2A protein or activity were studied in many sub-

cellular fractions which were prepared and studied by different methods, with different sample normalization procedures, and detection techniques used leading to divergent interpretation [Sim et al., 1994; Saito et al., 1995; Sontag et al., 1995; Strack et al., 1997; Price et al., 1999; Turowski et al., 1999]. However, association of PP2A and MTs was also described by others in rat or bovine brain tissues [Strack et al., 1997; Price et al., 1999; Hiraga and Tamura, 2000] based on cell fractionation, and Western immunoblotting analysis.

In contrast to PP2A, but consistent with our previously published observations [Verin et al., 1995], PP1 was presented in cytosolic as well as in several cytoskeletal fractions. PP2B was more abundant in actin- and vimentin-enriched EC fractions, which is also consistent with our previously published data indicating tight association of significant PP2B pool with detergent-insoluble EC cytoskeleton [Verin et al., 1998c]. Taken together, these data demonstrate differential subcellular distribution of PP1, PPase 2A, and 2B in endothelium and confirm specific and tight association of PP2A with MTs.

Several proteins which bind to both MTs and microfilaments have been identified and include MT-associated proteins (MAP1B, MAP2, tau) and the regulatory cytoskeletal protein, caldesmon [Satillaro et al., 1981; Ishikawa et al., 1992; Moraga et al., 1993; Ferhat et al., 1996; Togel et al., 1998]. It has became well established that MAPs control MT polymerization/depolymerization and are critically involved in the ability of MTs to respond to diverse signals such as those that induce cell division and cell motility. Importantly, reversible phosphorylation was shown to be a key event in the biological functioning of MAPs as primary targets for the regulation of MT structure [Drewes et al., 1998]. Using MT polymerization/depolymerization combined with differential centrifugation steps we demonstrated that significant amounts of tau, and HSP27 along with PP2A were present in the MT-enriched EC fraction suggesting the tight association of these proteins with MTs in endothelium, and the possible role of PP2A in the dephosphorylation of tau and HSP27. The association of HSP27 with tubulin/MTs in HeLa cells was reported [Hino et al., 2000]. HSP27, depending on its phosphorylation state, affects actin stress fiber and focal adhesion formation, two important components in EC barrier regulation [Benndorf et al., 1994; Gusev et al., 2002]. HSP27 is a terminal substrate for p38 MAPK cascade, and it was also demonstrated that PP2A dephosphorylates HSP27 in vivo [Cairns et al., 1994; Schneider et al., 1998; Armstrong et al., 1999; Gerthoffer and Gunst, 2001]. Experiments studying the role of PP2A-mediated HSP27 dephosphorylation in the alteration of MT and F-actin cytoskeleton in EC after various treatments are in progress.

MT-associated protein, tau, in its unphosphorylated form promotes assembly of MTs and inhibits MTs depolymerization [Drechsel et al., 1992]. Phosphorylation of tau by several kinases, including Ca^{2+}/calmodulin-dependent kinase II and protein kinase A, decreases its capability to bind MTs as well as to promote MT assembly [Drechsel et al., 1992; Singh et al., 1994; Gupta and Abou-Donia, 1999]. On the other hand, dephosphorylation of hyperphosphorylated tau restores its ability to promote MT assembly [Litersky et al., 1996]. Thus, biological function of tau is regulated by phosphorylation.

Our results show that the treatment of EC with PP2A inhibitor, OA, led to significant increase in tau phosphorylation confirming that tau is a substrate for 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 MTs [Drewes et al., 1998]. 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 MTs in the OA-treated EC. These data suggested the direct link between PP2A-mediated tau dephosphorylation and stability of peripheral MTs in EC. Recent studies demonstrate that specific isoforms of PP2A bind and dephosphorylate tau in neurons [Sontag et al., 1996]. Selective inhibition of PP2A by OA induced an Alzheimer-like hyperphosphorylation and accumulation of tau in mammalian brain. These studies also suggested that PP2A participated in the regulation of tau phosphorylation in vivo [Gong et al., 2000a]. Others show that reduced PP2A activity induces hyperphosphorylation and altered

compartmentalization of tau in transgenic mice [Kins et al., 2001].

The heterotrimeric form of PP2A, and more importantly the physiological substrates are involved in the PP2A-mediated EC barrier regulation are to be determined. However, our results indicate for the first time that PP2A, via one or more signaling pathways, may participate in the regulation of EC permeability.

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