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Caveolin regulates microtubule polymerization in the vascular smooth muscle cells

Jun-ichi Kawabe [a,c,*], Satoshi Okumura [a,d], Mark A. Nathanson [a], Naoyuki Hasebe [c], Yoshihiro Ishikawa [a,b,d,*]

[a] *Cardiovascular Research Institute, Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA*
[b] *Department of Medicine (Cardiology), University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, USA*
[c] *Department of Medicine, Asahikawa Medical College, Asahikawa 078-8510, Japan*
[d] *Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan*

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Abstract

Microtubule and caveolin have common properties in intracellular trafficking and the regulation of cellular growth. Overexpression of caveolin in vascular smooth muscle cells increased the polymer form of microtubule without changing in the total amount of tubulin, and downregulation of caveolin decreased the polymer form of microtubule. Fractionation of cellular proteins followed by immunodetection as well as immunostaining of caveolin and microtubule revealed that caveolin and a portion of microtubule were co-localized in caveolar fractions. A caveolin scaffolding domain peptide, which mimics caveolin function, did not alter the polymerization of microtubule in vitro, but dramatically inhibited the depolymerization of microtubule induced by stathmin, a microtubule destabilizing protein, which was also found in caveolar fractions. Accordingly, it is most likely that caveolin increased the polymer form of microtubule through the inhibition of a microtubule destabilizer, stathmin, suggesting a novel role of caveolin in regulating cellular network and trafficking.

Keywords: Scaffolding domain peptide; Stathmin; Depolymerization

Vascular smooth muscle cells (VSMCs) proliferation and migration are early pathological events leading to neointimal formation following acute or chronic arterial injury. Recently, several studies have suggested that caveolin, an important structural component of the caveolae, may play a critical role in the regulation of cell proliferation and migration [1,2]. Caveolins are generally downregulated in tumor cells [2], and upregulated in senescent cells [3]. Notably, the number of caveolae was dynamically reduced when VSMCs shift from a contractile to a proliferative phenotype, changing their configuration [4,5]. This phenotype-dependent variation in the expression of caveolae may be functionally important. However, molecular mechanisms in which caveolae regulate cellular proliferation or migration are poorly understood. Caveolin acts as a scaffolding protein to organize and concentrate a variety of signal molecules in caveolae, such as EGF receptors, G-proteins, Src-like kinases, Ras, and protein kinase C [6,7]. Interaction of caveolin with these molecules is generally inhibitory and thus caveolin may play a negative regulatory role in mitogenic signal transduction [6,8]. Accordingly, targeted downregulation of caveolin induces cell transformation through the hyperactivation of extracellular signal-regulated kinase (ERK) signal cascade [1]. In contrast, overexpression of caveolin attenuates signaling from EGF receptors, Raf, MEK-1, and ERK to the nucleus [9].

Caveolae have also unique endocytotic properties [7,10]. It is well known that caveolae are dynamic structure which travels between the cell surface and cellular-interior sites

* Corresponding authors. Fax: +1 973 972 7489.
E-mail addresses: kawabeju@asahikawa-med.ac.jp (J. Kawabe), ishikayo@umdnj.edu (Y. Ishikawa).

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with various stimuli, and this process requires an intact cytoskeletal network [11–13]. Caveolae are found in actin-rich regions of the cell membrane, which also contain a number of actin-binding proteins. Recently, it was demonstrated that an actin-binding protein, filamin, is directly associated with caveolin-1 [14]. It is thus possible that caveolin plays a role in organizing and regulating the function of cytoskeletons as well. The cytoskeletal components, such as microtubules, are of particular interest, because they have been implicated in the regulation of hormonal responses, cellular motility, intracellular organization, and transport [15]. The microtubules exist in a dynamic steady-state with α/β tubulin heterodimer, forming non-covalent polymer form, and their structure is subject to constant and dynamic remodeling. Depolymerization of microtubules can stimulate cell proliferation in the absence of other signals [16], whereas stabilization of microtubules can inhibit the action of mitogens [17]. It is also known that several signaling molecules, such as G-proteins [18,19] or ERK [20], are associated with tubulin or microtubules and these interactions may regulate their signaling cascades by themselves. Indeed, it has been shown that microtubule-disrupting drugs can attenuate signaling cascades such as Gs-cAMP [21] or Raf-ERK- and Src-like kinases pathways [22].

In this study, we have examined (1) whether changes in caveolin expression lead to alteration in microtubule, in particular, its polymerization, (2) whether caveolin associates with microtubules, and (3) potential molecular mechanisms of caveolin-mediated regulation of microtubule polymerization in VSMCs. We will thus demonstrate that caveolin regulates the polymerization of microtubule through the regulation of stathmin, a recently identified molecule that destabilizes microtubule [23,24].

Materials and methods

Cell culture and preparation of microtubule cytoskeleton. VSMCs were isolated from thoracic aorta of rats as described previously [25]. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Cells were grown to subconfluence and made quiescent by incubation in medium containing 0.5% FBS and 0.05% bovine serum albumin for 48 h. Polymerized microtubule specimen was prepared by washing the cells with MSB containing 0.1% Triton X-100 for 10 min at 25 °C as described [20]. The residual cytoskeleton was used as polymer microtubule specimen and the MSB extracts were used as monomer specimen of microtubule.

Adenoviral constructs. The adenoviruses encoding rat caveolin-1 and caveolin-3 were constructed using Adeno-X Expression system (Clontech, Palo Alto, CA) [26,27]. For downregulation of caveolins, the full-length cDNA encoding rat caveolin-1 and -3 were inserted in the antisense orientation in the shuttle vector and then the constructs were ligated to Adeno-X viral DNA. The linearized recombinant viral DNA was transfected into low-passage HEK293 cells. The recombinant virus was prepared according to the manufacturer's protocol. To control for adenoviral infection, an adenovirus encoding green fluorescent protein (GFP) was also used. The multiplicity of viral infection (MOI) for each virus was determined by dilution assay in HEK293 cells. According to the optimized condition using adenovirus encoding GFP (adeno-GFP), more than 90% of cells were infected.

Immunocytochemistry and immunoblotting. Cells were extracted as described above with MSB containing 0.1% Triton X-100 before the fixation with formaldehyde. Immunofluorescence staining of cytoskeleton was carried out as described [28]. Samples were incubated with primary antibodies at 4 °C for 18 h, followed by incubation with fluorescein isothiocyanate (FITC)- or rhodamine-conjugated second antibodies. The stained cells were viewed by epifluorescence microscopy and laser-scanning confocal microscopy. Immunoblotting was performed as described [28,29], and band density was quantitated by densitometry. Anti-caveolin-1, -3 antibodies were purchased from Transduction Labs. (San Diego, CA) and anti-α-tubulin antibody was from Sigma (St. Louis, MI).

Sucrose gradient fractionation. Caveolae fraction was separated from VSMCs by previously optimized sucrose gradient centrifugation method [29,30]. Among twelve fractions, the fractions 3–4 were used as caveolae fractions and fractions 5–12 were as non-caveolae fractions.

In vitro microtubule polymerization and depolymerization assay. Purified tubulin and recombinant stathmin were purchased from Cytoskeleton (Denver, CO) and Calbiochem-Novabiochem International, Inc (La Jolla, CA), respectively. These assays were performed as described [31] with modification. For polymerization reaction, tubulin (2.5 mg/mL) was mixed with buffer (in mmol/L; 80 Pipes, pH6.5, 1 EGTA, 0.5 $MgCl_2$, and 1 GTP) and incubated at 37 °C. For depolymerization assay, the mixture was incubated at 25 °C in the presence of stathmin (0.2 mg/mL). The polymerized tubulin was determined by measuring the absorbance at 340 nm using spectrophotometer. Synthesized peptides (the caveolin-1 scaffolding domain peptide; amino acid residues 82–101, and the caveolin-1 non-scaffolding domain peptide; amino acid residues 53–81) were prepared as described previously [31] and added into the reaction mixture at the concentration of 10 μmol/L.

Statistical analysis. The data are presented as means ± SEM. Statistical analyses of the data were performed using one-way ANOVA.

Results

Overexpression of caveolins enhanced the polymerization of microtubule

We first examined if the overexpression of caveolin changes the amount of microtubule polymerization in VSMCs. We infected cells with adenovirus harboring either caveolin-1 (adeno-Cav1) or caveolin-3 (adeno-Cav3) [26]. Two days after infection, caveolin was overexpressed by 10–20-fold. In contrast, infection of cells with adenovirus harboring GFP (control, adeno-GFP) did not alter the amount of endogenous caveolins (Fig. 1). Cells overexpressing caveolin showed an increased polymerization of tubulin while the amount of monomer form of tubulin was decreased in comparison to non-infected or control cells (Fig. 1). The amount of endogenous tubulin remained unchanged (data not shown).

Microtubule polymerization in response to caveolin overexpression was examined by immunostaining as well. The soluble monomer form of tubulin was extracted and the remaining polymer form of tubulin was stained in VSMCs (Fig. 2). Using FITC-conjugated secondary antibody, caveolin was detected in a punctuated pattern in both control cells and adeno-Cav1-infected cells, but the staining was much stronger in adeno-Cav1-infected cells (Fig. 2-C versus -A). Polymerization of tubulin, as evaluated using rhodamine-conjugated antibody, was enhanced in adeno-Cav1-infected cells (Fig. 2-D versus -B), consistent with the results from immunoblotting experiments (Fig. 1). The staining of polymer form of tubulin was

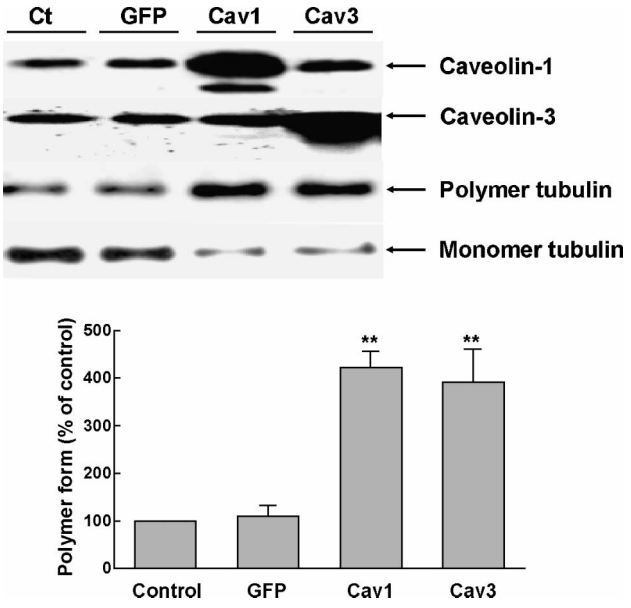

Fig. 1. Caveolin overexpression and microtubule polymerization. VSMCs were infected with adenovirus encoding caveolin-1 (*Cav1*) or caveolin-3 (*Cav3*) at 50 MOI. Non-infected (*Ct*) or virus encoding GFP-infected cells (*GFP*) were used as control. Forty eight hours after infection, polymer and monomer tubulin were separated and quantitated by immunoblotting. Representative immunoblottings after separation with 4–20% SDS–PAGE are shown. Bar graphs show the relative amount of the polymer form of tubulin from four independent experiments (mean ± SEM, $^{**}p < 0.01$). The level of polymer tubulin of control was set at 100%.

subsided when the cells were treated with nocodazole (5 μmol/L), which destroys the polymer form of tubulin (data not shown).

Downregulation of caveolins attenuated the polymerization of microtubule

The above experiments demonstrated that an increase in caveolin expression led to enhanced polymerization of tubulin. To test the role of endogenous caveolin in the microtubule polymerization, caveolin was then downregulated using adenovirus harboring antisense caveolin-1 (adeno-antiCav1) or -3 (adeno-antiCav3) as described previously [26]. In antisense caveolin infected VSMCs, endogenous caveolin was decreased to 30%–40% of that in control cells and this attenuation occurred in a caveolin-subtype specific manner (Fig. 3). However, regardless the subtype of caveolin downregulated, the amount of polymerized microtubule was decreased. Again, the total amount of endogenous tubulin was not changed (data not shown). Putting together, it appears that the amount of polymerized tubulin changes was regulated by the amount of caveolin.

Tubulin was found in caveolae

It has been shown that caveolin acts as scaffolding protein. Caveolin-associated proteins, including G-proteins

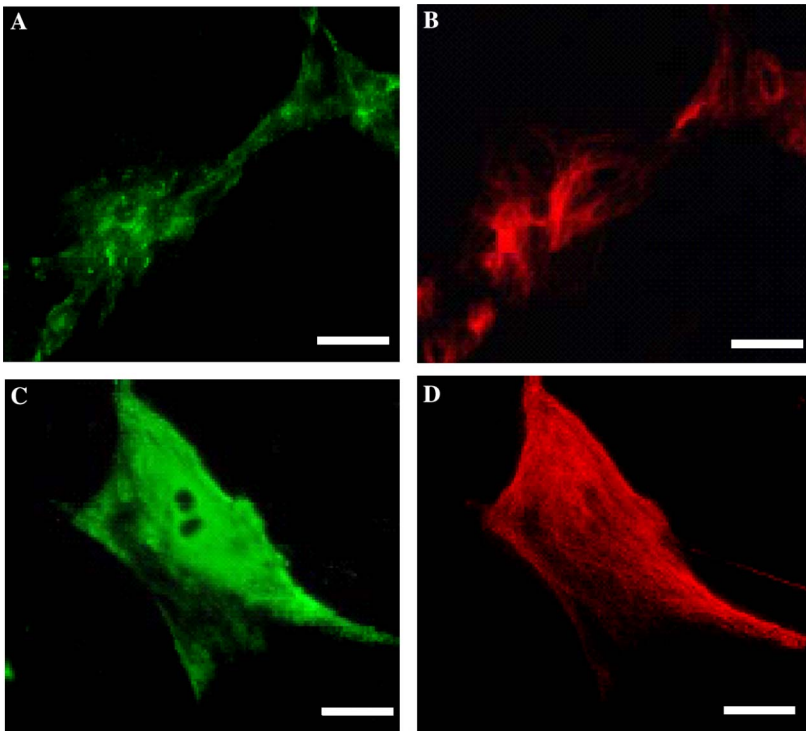

Fig. 2. Immunostaining of caveolin-1 and polymerized microtubule in VSMC. VSMCs were infected with adenovirus harboring caveolin-1 (C,D) or non-infected (A,B). Forty-eight hours after infection, monomer tubulin was extracted from the cells. Subsequently, caveolin-1 (A,C) and the polymer form of α-tubulin (B,D) were double-stained and visualized using anti-FITC (left, green) and anti-rhodamine (right, red)-conjugated antibodies, respectively. Bar indicates 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

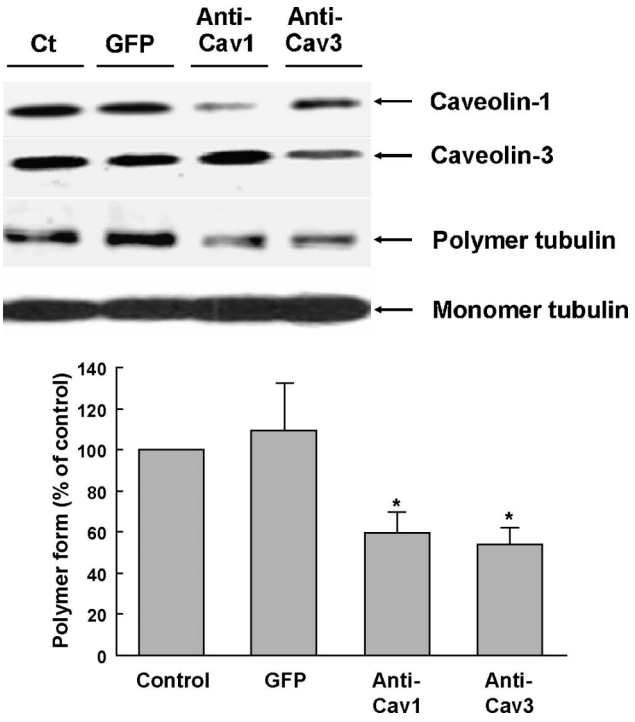

Fig. 3. Downregulation of caveolin and microtubule polymerization. VSMCs were infected with adenovirus encoding antisense caveolin-1 (anti-*Cav1*) or antisense caveolin-3 (anti-*Cav3*) at 50 MOI. Non-infected (*Ct*) or virus encoding GFP-infected cells (*GFP*) were used as control. Forty-eight hours after infection, polymer and monomer tubulin were separated and quantitated by immunoblotting. Representative immunoblottings after separation with 4–20% SDS–PAGE are shown. Bar graphs show the relative amount of the polymer form of tubulin from four independent experiments (mean ± SEM, $^{*}p < 0.05$). The level of polymer tubulin of control was set at 100%.

and adenylyl cyclase, can be co-purified within the caveolae fractions by the sucrose gradient fractionation method [32,33]. In order to examine if caveolin and tubulin can be co-localized in cells, sucrose gradient centrifugation of the homogenates of VSMCs and subsequent immunoblot analyses were performed. As shown in Fig. 4, caveolin was predominantly found within the fractions 3/4, which are known to be caveolar fractions [29,30]. A part of tubulin was also found within these fractions although tubulin was detected mostly in non-caveolar fractions 7–12. We also performed double immunostaining of caveolin and polymerized tubulin and analyzed with a confocal microscope. As shown in Fig. 5, caveolin and polymerized microtubule were co-localized, at least partially, within the plasma membrane. These results suggest that a part of caveolin is co-localized with tubulin.

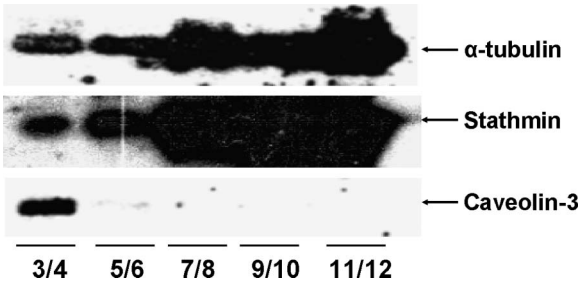

Fig. 4. Subcellular localization of α-tubulin, stathmin, and caveolin-3. VSMCs were homogenated and subjected to sucrose gradient centrifugation, and caveolae fractions (*3/4*) and non-caveolae fractions (*5/6*, *7/8*, *9/10*, and *11/12*) were separated. An aliquot from each sample was separated by 4–20% SDS–PAGE and analyzed by immunoblotting for α-tubulin, stathmin, and caveolin-3. Representative immunoblottings after separation with 4–20% SDS–PAGE are shown.

Caveolin peptide inhibited stathmin

In order to examine whether caveolin by itself regulates the microtubule polymerization, we performed microtubule polymerization assays in vitro using a caveolin-1 scaffolding domain peptide (CSDP), which is known to mimic caveolin-1 protein in regulating various enzyme activities [30,34]. Incubation of monomer tubulin with GTP led to the formation of polymer tubulin within 20 min and the formed polymer was stable, at least, for up to 30 min. Addition of CSDP, however, did not alter the microtubule polymerization and also did not affect the stability of polymerized tubulin, suggesting that caveolin has no direct effect on microtubule (data not shown).

Stathmin/Op18 is a small, heat-stable protein that was recently identified as microtubule destabilizing factor [23,24]. We found that stathmin was found in caveolae fractions (Fig. 4), suggesting stathmin co-exists with caveolin in caveolae. We therefore postulated that caveolin may regulate the activity of stathmin and thus the state of microtubule polymerization. As shown in Fig. 6, in vitro microtubule depolymerization assays demonstrated that stathmin indeed depolymerized tubulin. Within 10 min after the addition of stathmin, the amount of polymer microtubule was decreased by half (Fig. 6). In the presence of CSDP in the reaction mixture, however, stathmin-induced depolymerization was inhibited, suggesting CSDP inhibited the activity of stathmin. When a caveolin peptide derived from a non-scaffolding domain was used, there were no changes in the stability of microtubule polymer and the stathmin-induced depolymerization of microtubule.

Discussions

We have demonstrated that the amount of caveolin is an important factor to regulate the state of polymerization of microtubule in VSMCs. Overexpression of caveolin led to increased polymer form of microtubule while downregulation of caveolin decreased the polymer form. Because the total amount of tubulin was unchanged by either caveolin overexpression or downregulation, it was most likely that the polymerization reaction and/or the stability of polymer form of microtubule were altered. In support of the former concept, we have demonstrated that caveolin scaffolding domain peptide inhibited the stathmin-mediated depolymerization of microtubules. Interestingly, the same peptide had no direct effect on

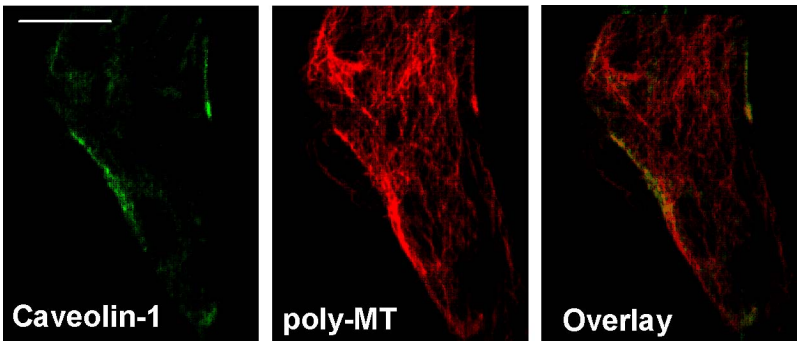

Fig. 5. Co-localization of caveolin and microtubule in VSMC. Caveolin-1 and the polymer form of α-tubulin were double-stained after monomer tubulin was extracted, and visualized using anti-FITC (*Caveolin-1*, green) and anti-rhodamine (*poly-MT*, red)-conjugated antibodies, respectively. Overlayed image is also shown (*Overlay*). Bar indicates 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

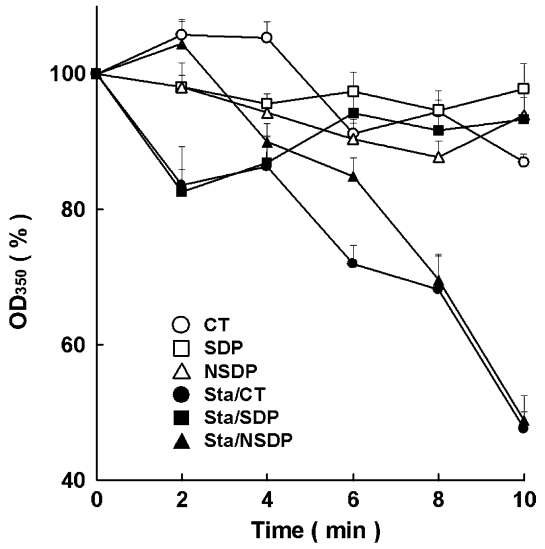

Fig. 6. Effect of caveolin peptide on stathmin-induced microtubule depolymerization. Polymerized tubulin was incubated at 25 °C in the absence or presence of stathmin (*Sta*) and/or caveolin peptides (*SDP*, caveolin-1 scaffolding domain peptide; *NSDP*, caveolin-1 non-scaffolding domain peptide; *CT*, no peptide). Polymer form of tubulin was quantitated by measuring the absorbance at 340 nm (mean ± SEM, $n = 6$).

microtubule itself. Thus, caveolin enhanced the polymerization of microtubule most likely through the inhibition of stathmin.

Caveolin has been known as a major inhibitor of cellular signal; in most cases, caveolin directly inhibits the activity of enzymes and/or kinases directly involved in cellular signal cascades [35]. In this study, however, we propose an additional role of caveolin, i.e., caveolin plays an important role in regulating the cellular traffic network of microtubule, which, indirectly, contributes to the regulation of cellular signaling. It is now well known that microtubules contribute not only to the cellular morphology, but to the regulation of signal transduction itself (for review see [15]). Many signal molecules are indeed associated with the along of microtubules and thereby depend on microtubule. For example, caveolin inhibits ERK, one of the most potent mitogenic signal molecules, at the level of caveolae in the plasma membrane. However, activated ERK needs to translocate into the nucleus, where ERK stimulates a series of transcriptional factors to transmit mitogenic signal [36]. ERK is associated with microtubules, and the separation of the microtubule-bound ERK is shown to prevent its nuclear translocation in a previous study [20]. Similarly, caveolin directly inhibits the activity of Gs protein [6]. Microtubules associate with Gs protein and are involved in transmitting Gs protein signal to adenylyl cyclase as demonstrated by the attenuation of cAMP production with disruption of microtubule [37]. These findings support the concept that caveolin regulates cellular signaling not only at the level of signaling molecule, but at the level of intracellular traffic as well.

The polymerization dynamics of microtubules are central to their biological functions. Polymerization dynamics allow microtubules to adopt spatial arrangements that can change rapidly in response to cellular needs and, in some cases, to perform mechanical work. Microtubules can also function as rigid struts opposing the force generated by actin/myosin interaction and therefore may contribute to cytoskeletal stiffness and also cellular contractions as well [15,38]. Putting together, the role of caveolin, at least in VSMCs, may not be restricted to directly regulating the molecules involved in cellular signal, but to regulate the status of polymerization of microtubules, which indirectly regulates cellular signal. It is tentative to speculate that such changes would contribute to altering the vascular mechanical properties, such as cellular stiffness and contractility, through the altered states of microtubule polymerization under pathophysiological conditions as well.

Acknowledgments

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