Alteration of microtubule polymerization modulates arteriolar vasomotor tone

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Platts, Steven H., Jeff C. Falcone, William T. Holton, Michael A. Hill, and Gerald A. Meininger. Alteration of microtubule polymerization modulates arteriolar vasomotor tone. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H100–H106, 1999.—Microtubules are important cytoskeletal elements that have been shown to play a major role in many cellular processes because of their mechanical properties and/or their participation in various cell signaling pathways. We tested the hypothesis that depolymerization of microtubules would alter vascular smooth muscle (VSM) tone and hence contractile function. In our studies, isolated cremaster arterioles exhibited significant vasoconstriction that developed over a 20- to 40-min period when they were treated with microtubule depolymerizing drugs colchicine (10 µM), nocodazole (10 µM), or demecolcine (10 µM). Immunofluorescent labeling of microtubules in cultured rat VSM revealed that both colchicine and nocodazole caused microtubule depolymerization over a similar time course. The vasoconstriction was maintained over a wide range of intraluminal pressures (30–170 cmH2O). The increased tone was not affected by endothelial denudation, suggesting that it was due to an effect on VSM. Microtubule depolymerization with demecoline or colchicine had no effect on VSM intracellular Ca2+ concentration ([Ca2+]i). These data indicate that microtubules significantly interact with processes leading to the expression of vasomotor tone. The mechanism responsible for the effect of microtubules on vasomotor tone appears to be independent of both the endothelium and an increase in VSM [Ca2+]i.

METHODS

Isolated Vessel Preparation

Male Sprague-Dawley rats (250–350 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). Anesthesia was confirmed by the loss of spinal reflexes. One cremaster muscle was carefully removed and placed in a refrigerated (4°C) lexan chamber filled with physiological saline solution (PSS) containing (in mM) 145 NaCl, 4.7 KCl, 3 CaCl2, 1.2 MgSO4, 1.0 NaH2PO4, 5.0 dextrose, 2.0 pyruvate, 0.02 EDTA, 3.0 MOPS, and 0.15 albumin. A first-order segment of the cremaster feed arteriole was isolated, removed, and placed in a separate bathing chamber. The vessel bath chamber was filled with a HCO3−-buffered PSS containing (in mM) 110 NaCl, 5 KCl, 3 CaCl2, 1 MgSO4, 1 KH2PO4, 0.02 EDTA, and 30 NaHCO3 that was equilibrated with 5% CO2–95% N2. This chamber was fitted with pipette holders, and the entire apparatus was mounted on an inverted microscope stage plate. Isolated arterioles were cannulated with two glass micropipettes (80- to 100-µm OD). The proximal end of the arteriole was tied to an open pipette using 12-0 nylon suture, and any red blood cells were flushed from the lumen by application of gentle positive pressure (~10 cmH2O). The distal end of the vessel was then cannulated and tied to a dosed micropipette. The vessel-mounting apparatus was transferred to the stage of an inverted microscope (Zeiss IM35) and was slowly warmed to 34.5°C. Arterioles were pressurized to 90 cmH2O by adjusting the height of a water reservoir and were checked for leaks by turning off the pressure head for 30 s. Any vessels that
showed a decrease in diameter were discarded. At the conclusion of each experiment, adenosine (10^{-4} M) was added in a Ca^{2+}-free PSS containing 4 mM EGTA to obtain passive diameter at 90 cmH2O. Lumen diameters were recorded on-line using a video monitor coupled to a video caliper system (5). Myogenic responses were studied by exposing arterioles to step changes in intravascular pressure (20-40 cmH2O steps) over a range of 30-170 cmH2O. Diameter measurements were recorded for 4 min after each step to assure a steady-state response.

Manipulation of Microtubule Network Structure

After we obtained a control pressure-diameter curve, arterioles were treated for 1 h with nocodazole (10 µM, n = 4), colchicine (10 µM, n = 8), or demecolcine (10 µM, n = 9) to disrupt microtubules. All three of these inhibitors bind to the colchicine-binding site on tubulin and prevent addition of tubulin to the microtubule, leading to a net loss of polymerized tubulin (18). A second pressure-diameter relationship was then acquired. Demecolcine was prepared as a 5 mM stock solution with water and was diluted to the appropriate concentration immediately before each experiment. All other microtubule-modulating drugs were prepared as concentrated stocks with DMSO and were diluted with PSS to the appropriate concentrations.

Immunocytochemistry

Cells. To determine the effectiveness of the microtubule-depolymerizing agents, the microtubule network was examined in VSM after treatment with colchicine or nocodazole using fluorescent immunocytochemistry. VSM cells were enzymatically isolated from rat thoracic aorta. Briefly, aortic segments were stirred at 37°C for 45 min in an enzymatic solution containing (in mM) 118 NaCl, 6 KCl, 25 MOPS, 1.2 NaH2PO4, 1.2 MgSO4, 0.027 EDTA, 11 glucose, 2 dithiothreitol, and 2 mg/ml papain. After incubation, aortic segments were allowed to settle to the bottom of the vial, and the solution was carefully aspirated and replaced with culture media (DMEM). Vessel segments were then rapidly trituated in a test tube, and 2 ml of cold 0.1% Triton X-100. Cells were centrifuged, resuspended in media, and plated at a density of 1 x 10^5 cells/ml. Cells were grown in DMEM supplemented with 10% FBS and 2% (vol/vol) antibiotic/antimycotic. At passage 4, the cells were treated with trypsin (0.1%) and replated on glass coverslips, where they were allowed to attach and spread for 24 h. After colchicine treatment, the cells were fixed at 0, 15, 30, and 60 min with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with a mixture of monoclonal mouse anti-α-tubulin and anti-β-tubulin antibodies followed by incubation with a goat anti-mouse secondary antibody conjugated with FITC. Cells were visualized and imaged on a Zeiss Axiolab 200 coupled to a CellScan Digital Imaging Microscopy System (Scanalytics, Billerica, MA). All images were deconvolved to remove nonfocused light.

Arterioles. Isolated arterioles were incubated with nocodazole (0 µM, n = 2) for 60 min or vehicle (DMSO) as a control. After treatment, vessels were fixed in 2% paraformaldehyde and permeabilized with cold acetone (−20°C) for 1 min. All vessels were then incubated with a mixture of mouse FITC-conjugated anti-α-tubulin and anti-β-tubulin for 90 min in a humidified tissue culture chamber. Next, each arteriole was individually mounted on a glass microscope slide using the ProLong anti-fade kit (Molecular Probes, Eugene, OR). Labeling and imaging of the treated vessels was performed on the same day to minimize variations due to the labeling procedure. Vessels were imaged with an Ultima-Z argon laser confocal scanning microscope (Genomic Solutions, Lansing, MI). Fluorescent images were background subtracted, and intensity measurements were performed by drawing regions of interest in different sections of each vessel. The mean fluorescent intensity (scale of 0-4,095) was calculated and normalized to control vessels to allow for comparison between vessels.

α-Adrenergic Blockade

To determine whether microtubule depolymerization was causing release of norepinephrine from adrenergic nerve terminals, phentolamine (1 µM, n = 8) was used to block vascular α-adrenergic nerve terminals in the arteriolar wall. Vessels were pretreated with phentolamine for 20 min and then were subjected to the colchicine treatment protocol.

Endothelium Denudation

To test for the possible involvement of the endothelium, pressure-diameter relationships were acquired from colchicine-treated vessels before and after the endothelium was mechanically removed (n = 6). Denudation was performed by physically rubbing the intraluminal surface with an abrasive micropipette. Previous work in our laboratory has shown that this type of endothelial denudation has no effect on resting vascular tone (4). The absence of a functional endothelium was confirmed by the lack of dilation to ACh (1 µM).

VSM Ca^{2+}

To determine whether the vasconstriction due to microtubule disruption was caused by increased [Ca^{2+}]i, VSM [Ca^{2+}]i was measured with the Ca^{2+}-sensitive fluorescent indicator fura 2-AM (Molecular Probes) using previously described techniques (11). After equilibration, vessels were abuminally incubated with a suspension of fura 2-AM (2-5 µM), DMSO (0.5%), and pluronic F-127 (0.01%) in normal PSS for ~1 h. After being loaded with fura 2, the vessel was washed with fresh PSS and equilibrated for at least 30 min. Fura 2 fluorescence emissions were measured at 510 nm during excitations at 340 and 380 nm. The ratio of fura 2 fluorescence at 340 to 380 nm is directly proportional to [Ca^{2+}]i (6). In these experiments, the 340- to 380-nm ratio was determined before and after treatment with demecolcine (10 µM, n = 3) or colchicine (1.25 x 10^{-4} M, n = 8). A test dose of norepinephrine (5 mM) was applied at the beginning and end of each Ca^{2+} measurement experiment to verify the ability of the Ca^{2+} measurement system to detect changes in Ca^{2+}.

Data Analysis and Statistics

Data were normalized to passive diameter at 90 cmH2O and are expressed as means ± SE. Arteriolar responses were analyzed using ANOVA followed with either Dunnett’s test or Fisher’s protected least squares difference for multiple comparisons. Significance was defined with a probability <0.05.

RESULTS

All vessels studied gained spontaneous tone resulting in a reduction in diameter to 50-70% of passive diameter. Mean arteriolar diameter with spontaneous tone was 114 ± 4 µm at 90 cmH2O. Colchicine treatment significantly increased vasomotor tone (33.9% constriction relative to basal tone at 90 cmH2O) within 20-40 min as did all other microtubule-disrupting agents. Figure 1 shows the pressure-diameter curves for arterioles in control and treated conditions.
before and after treatment with $10^{-5}$ M colchicine as well as the corresponding passive diameter curve. Using 90 cmH$_2$O as a reference, the control diameter in vessels with spontaneous tone was $70 \pm 6\%$ of passive diameter. After treatment with colchicine, arterioles constricted to $48 \pm 3\%$ of passive diameter. This constriction was maintained over the entire pressure range and was significantly different from both the control diameter and the passive diameter. As shown in Fig. 2, nocodazole also caused significant vasoconstriction of arterioles with spontaneous tone (29.6% at 90 cmH$_2$O). Similarly, demecolcine produced a significant vasoconstriction (32.7%) in vessels with spontaneous tone (Fig. 3). Thus all of the microtubule-depolymerizing drugs were found to affect spontaneous vasomotor tone in a similar manner, reducing the possibility that the constriction was due to a nonspecific action of the drug treatments.

Various concentrations of demecolcine ($0.1$–$100$ µM) were used to obtain a concentration-response relationship ($n = 17$). In addition, vessels ($n = 7$) were treated with $10$ µM demecolcine, and diameter measurements were obtained throughout the 60-min incubation period to obtain a response time course. Figure 4A shows the concentration-response relationship for demecolcine, in which $>90\%$ of the vasoconstriction associated with demecolcine occurred with a concentration of 10 µM. With this concentration of demecolcine, vessels were observed to constrict throughout the 60-min time period, reaching maximal levels of constriction between 40 and 60 min (Fig. 4B).

Microtubule disruption was confirmed with immuno-cytochemical experiments. Both colchicine ($10$ µM) and nocodazole ($10$ µM) caused time-dependent disassem-
not shown) also showed decreased microtubule density, as indicated by a 45 ± 0.15% reduction in fluorescent intensity of labeled microtubules compared with control. Thus the time course observed for depolymerization of microtubules approximated the time curve of the vasoconstrictor response (Fig. 4B).

To determine whether the vasoconstriction observed with the depolymerizing agents was the result of release of a vasoactive endothelial factor, we mechanically removed the endothelium from six vessels with an abrasive pipette. These vessels were then treated with colchicine and were observed to undergo vasoconstriction in response to colchicine similar to that of vessels with intact endothelium (Fig. 6).

Another group of vessels (n = 8) was treated with phentolamine (1 µM) to antagonize α-adrenergic receptors. Phentolamine treatment had no effect on colchicine-induced vasoconstriction (46.2 ± 3.3 vs. 44.3 ± 2.7% of passive diameter), indicating that the constrictor response was not due to an effect of microtubule depolymerization on adrenergic nerve terminal release of norepinephrine. However, these experiments do not rule out the possibility of release of a vasoactive cotransmitter.

To determine whether a change in VSM [Ca2+]i was involved in this vasoconstriction, isolated arterioles were abuminally loaded with the Ca2+-sensitive fluorescent dye fura 2-AM (n = 11). The 340- to 380-nm ratio was used as a measure of VSM [Ca2+]i. Demecolcine-treated (10 µM, n = 3) arterioles exhibited a time-dependent increase in vascular tone with no concurrent change in [Ca2+]i (Fig. 7). Vessels (n = 8) treated with colchicine (1.25 × 10⁻⁵ M) produced similar results. After 1 h of incubation with colchicine, arterioles had a 340- to 380-nm fura ratio of 1.7 ± 0.01, which was not different from control vessels in which the ratio was 1.8 ± 0.11.

**DISCUSSION**

The purpose of this study was to test the hypothesis that the depolymerization of microtubules would alter VSM contractile function and thus the expression of vasomotor tone and vascular diameter. We observed that three different microtubule-disrupting drugs caused a significant vasoconstriction. Colchicine, nocodazole, and demecolcine all caused significant arteriolar constrictions (33.9, 29.6, and 32.7% relative to basal tone, respectively) that were not statistically different from each other. We also confirmed that both colchicine (Fig. 5) and nocodazole (data not shown) caused complete disruption of the microtubule network in cultured VSM cells over a time course similar to that observed for the appearance of the vasoconstriction, suggesting that the two events are correlated. This constriction was not attributed to activation of α-adrenergic nerve terminals in the vascular wall, release of a vasoactive endothelial substance, or an increase in VSM [Ca2+]i.

A role for microtubules in modulating contractile function has been previously shown in both nonmuscle and muscle cells. Danowski (3) and Kolodney and Elson (9) have independently demonstrated that fibroblasts contracted when microtubules were disrupted with demecolcine, vinblastine, and/or nocodazole. Both groups used in vitro assays of fibroblast contraction, but Danowski measured deformation (wrinkling) of a silicone substrate, whereas Kolodney and Elson measured isometric force generated from cells cultured within a collagen lattice. Additionally, they found that paditaxel could reverse the contraction (2, 9). Thus they concluded that the modulation of contraction/force generation was microtubule dependent. In cardiac myocytes, Tsutsui et al. (16) showed that microtubule density was increased during pressure-induced cardiac hypertrophy and that observed contractile deficiency correlated with the increase in microtubule density. Cells treated with paditaxel to increase microtubule density exhibited a similar degree of contractile deficiency. This dysfunction could be reversed by treatment with colchicine or cold, both of which depolymerize microtubules. It was concluded from their studies that microtubules impose a viscous intracellular load and...
that increasing microtubule density increased this viscous load, thus opposing myocyte contraction. Thus these studies support a role for microtubules/tubulin in modulating cellular contractile function.

Recently, several investigators have begun to examine the effect of microtubule depolymerization on VSM contraction. Sheridan et al. (15) showed that agonist-dependent contraction of rings from the pulmonary artery was significantly increased after treatment with the microtubule-depolymerizing agent vinblastine. Similarly, Leite and Webb (10) observed an enhanced vasoconstriction to $\alpha$-adrenergic receptor activation in mesenteric arteries treated with nocodazole. Another study, in aortic VSM rings, showed that complete microtubule disruption with colchicine and/or cold enhanced stretch-induced tension development (2). Similarly, our results showed that microtubule depolymerization enhanced development of vasomotor tone in pressurized resistance arterioles. This increase in vasomotor tone was superimposed on intrinsic spontaneous tone and was maintained throughout the myogenic pressure range. However, it is premature to define this augmented tone as myogenic in nature without a more detailed understanding of the mechanism. It was also apparent in our studies that the enhanced tone was not accompanied by augmented myogenic responses to step changes in pressure. Collectively, the changes in vasomotor tone and vascular reactivity reported by these studies establish a clear link between microtubules/tubulin and the VSM contractile process. However, the mechanism responsible for the relationship between microtubules and the contractile process remains undefined.

Several mechanisms may be proposed to explain the ability of microtubules to modulate contractile function. Generally, these can be described as belonging to one of the following four groups: 1) those involving an effect on nerve terminals within the vessel wall, 2) involvement of endothelial cell vasoactive factor(s), 3) changes in cellular signaling mechanisms, or 4) mechanisms related to mechanical properties of the cytoskeleton. In our study, phentolamine antagonism of $\alpha$-adrenergic receptors was without effect, indicating that norepinephrine release was not involved. This observation does not, however, rule out the possibility that there may be release of a vasoconstrictive cotransmitter.
Removal of the endothelium did not have any effect on the vasoconstriction caused by microtubule depolymerization, suggesting that the endothelium is not essential in mediating this response. In mesenteric arteries, however, Leite and Webb (10) reported that nitric oxide synthase inhibition with \( \text{N}^\text{G}\)-nitro-L-arginine potentiated pressor responses after nocodazole treatment. These differences point to possible tissue-specific differences and suggest that a modulatory role for the endothelium cannot be entirely dismissed.

Numerous chemical mediators have been proposed to account for the cellular effects related to the state of microtubule polymerization. Rasenick and Wang (14) have proposed that transduction of signals in neurons via adenylylate cyclase may be mediated by tubulin monomers, which are proposed to interact with G proteins by virtue of their ability to reversibly bind GTP. More recently, Popova et al. (13) have shown that tubulin can regulate PLC activity. The regulation of PLC appears complex, as low cellular concentrations of tubulin activated PLC, whereas high concentrations inhibited PLC activity. An additional possibility was proposed by Kolodney and Elson (9). They showed that microtubule disruption in fibroblasts leads to increased phosphorylation of the myosin regulatory light chain. Although the mediator for this phosphorylation event was not identified, it could be related to regulation of \([\text{Ca}^{2+}]\). Collectively, phosphorylation of the myosin regulatory light chain and the adenylylate cyclase and PLC signaling systems is known to be associated with regulation of the contractile apparatus and thus bears further investigation in VSM.

Because \([\text{Ca}^{2+}]\) could be related to both phosphorylation of the myosin regulatory light chain and PLC activation, we examined the possibility that microtubule disruption could be related to regulation of VSM \([\text{Ca}^{2+}]\). Using the \(\text{Ca}^{2+}\)-sensitive dye fura 2, we were unable to detect any change in cell \([\text{Ca}^{2+}]\) after treatment with demecolcine or colchicine. This is in agreement with published data in cardiac myocytes (16) in

![Fig. 7. Estimates of vascular smooth muscle intracellular \([\text{Ca}^{2+}]\) concentration ([\(\text{Ca}^{2+}\)]i) in intact arterioles before and after demecolcine (10 \(\mu M\)) treatment using fura 2-AM. A: representative tracings of 340- to 380-nm ratio (top) and arteriolar diameter (bottom). Arrows labeled NE represent additions of norepinephrine (5 \(\mu M\)) as positive controls for viability and increased [\(\text{Ca}^{2+}\)]. B: average responses of arterioles (n = 3) for 340- to 380-nm ratio (left) and intraluminal diameter (right). Bar graphs show that, although demecolcine causes vasoconstriction, there is no change in [\(\text{Ca}^{2+}\)]. *\(P < 0.05\) and **\(P < 0.01\).]
which colchicine actually caused a small decrease in [Ca\(^{2+}\)]. The lack of a change in [Ca\(^{2+}\)] suggests that the putative involvement of the above signaling pathways in microtubule-induced contraction may be more complex in nature.

Perhaps the best-known theory incorporating and integrating the mechanical properties of the cytoskeleton with cellular function is the "tensegrity" theory of cellular architecture (7, 8). In this model, microtubules are components of the cytoskeleton believed to act as compression-resistant elements within the cell. These elements are proposed to oppose the contractile "pull" of the actin cytoskeleton. On the basis of this model, it has been speculated that disrupting the microtubules (compression-resistant struts) would act to reduce the internal mechanical resistance to contraction. Thus the tension-generating elements (i.e., contractile apparatus) would be able to shorten more efficiently. This could translate into increased force generation and contraction of the cell. Consequently, this mechanism may also be a possible explanation for the vasoconstriction we observed after microtubule depolymerization.

In conclusion, our data support the hypothesis that arteriolar smooth muscle contractile function and arteriolar diameter can be modulated by the state of polymerization of the microtubule network. It may be that cytoskeletal remodeling can provide an additional mechanism for modulating VSM contractile function without a requirement for simultaneously increasing [Ca\(^{2+}\)]. Although the mechanism for this effect remains to be defined, these observations could have significant implications for our understanding of the physiological and pathophysiological regulation of VSM contractile function.

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