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Endothelial cytoskeletal elements are critical for flow-mediated dilation in human coronary arterioles

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Abstract

Mitochondrial H_2O_2 contributes to flow-mediated dilation (FMD) in human coronary arterioles (HCA). We examined the hypothesis that the endothelial cytoskeleton plays a critical role in transducing endothelial wall shear stress into a stimulus for releasing mitochondrial ROS. Phallacidin together with α-, β-tubulin antibodies and Mito-Tracker Red showed the proximity of F-actin, microtubules and mitochondria in endothelial cells. Cytochalasin D (CytoD) and nocodazole (Noc) disrupted endothelial F-actin and microtubules in HCA, respectively, concurrent with a reduction in the generation of cytosolic $O_2^{\bullet-}$ and H_2O_2 (hydroethidine and dichlorodihydrofluorescein fluorescence) and mitochondrial superoxide (mitoSox) during flow (control: 3.5 ± 1.6, Cyto D: 0.51 ± 0.2, Noc: 0.81 ± 0.6). FMD, but not the dilation to bradykinin or papaverine, was reduced by Cyto D (26 ± 10% vs. 56 ± 3%) or Noc (26 ± 11% vs. 58 ± 7%). These results suggest that cytoskeletal elements are a critical component of the signaling mechanism linking endothelial shear stress and mitochondrial release of ROS in the human coronary microcirculation.

Keywords

Shear stress; Cytoskeleton; Coronary microcirculation; Oxidative stress

1 Introduction

Vasomotor tone is regulated by a variety of chemical and physical factors. One of the most important physiological means of eliciting dilation is by increasing flow through an artery or arteriole. The resulting change in wall shear stress on the vascular endothelium elicits a complex signaling process that results in the release of factor(s) that traverse the basement membrane and act upon the underlying smooth muscle to elicit relaxation. The resulting vasodilation is termed flow-mediated dilation (FMD), is usually dependent on the endothelium,

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and is observed in most vascular beds [7,26,34]. It has been suggested that flow-mediated dilation provides continuity in the dilator response throughout a vascular bed by allowing communication between downstream resistance arterioles and upstream feeder conduit vessels. For example, exercising muscle releases vasodilator metabolites that relax the local downstream resistance arterioles increasing tissue perfusion. The resulting increase in flow occurs in both the arterioles as well as in the upstream conduit vessels where it elicits a flow-mediated dilation that facilitates delivery of blood to the exercising muscle.

In animal models the mechanism of flow-mediated dilation (FMD) is traditionally thought to involve endothelial release of nitric oxide (NO) [8,9,21], prostaglandins [18,19] and cytochrome P450 (CYP450) metabolites of arachidonic acid [36]. Recent studies from our laboratory suggest that FMD occurs in coronary arterioles from patients with coronary disease (CAD) however it operates through a novel mechanism involving endothelial production of reactive oxygen species (ROS), specifically hydrogen peroxide (H_2O_2) [31,35]. H_2O_2 can hyperpolarize vascular smooth muscle [35] and has been demonstrated to be an endothelial derived hyperpolarization factor (EDHF) by other investigators [11,13,32]. The endothelial derived H_2O_2 responsible for FMD in the human heart was determined to originate from the mitochondrial respiratory chain [31]. However, it is not clear how mitochondria are involved in the transduction of the mechanical shear stress signal that ultimately elicits vasodilation.

Cytoskeletal elements are known to be involved in shear stress-induced endothelial signaling. Wall shear stress activates focal adhesion kinases linked to integrins on the abluminal membrane through actin filament connections [12,17]. Hutcheson et al. [20] showed that shear stress-induced NO-mediated dilation in rabbit aorta was dependent upon intact microtubules and filaments. Sun et al. [43] extended these observations to the microcirculation using rat gracilis arterioles. These investigators showed that nocodazole or colchicine disrupted endothelial microtubules and prevented flow-induced dilation. Most of these studies were performed in animals where FMD is regulated by non-EDHF mechanisms. It is not known whether a similar cytoskeletal signaling pathway exists in HCA where mitochondrial ROS formation is the predominant mechanism for FMD. The overall hypothesis of this study is that shear stress acting on endothelial cells through attached cytoskeletal elements results in mitochondrial production of superoxide ($O_2^{\bullet-}$) and H_2O_2 and dilation of HCA. The novel focus of this study is to determine if cytoskeletal structures can link cell processes including mechanotransduction, mitochondrial respiration and redox signaling to regulate flow induced dilation.

2 Materials and methods

2.1 Cultured endothelial cells

Human coronary arterial endothelial cells (HCAEC) purchased from Clonetics were seeded and incubated in endothelial cell growth medium (EGM-2-MV; Clonetics). When 70-80% confluence was achieved, cells were subcultured and plated onto 10 × 20 mm coverslips to the same confluency for immunofluorescence.

2.2 Immunocytochemistry

HCAECs were incubated with culture medium containing 100 nM Mito Tracker Red at 37 °C for 30 min. After washing, cells were fixed with 3.5% paraformaldehyde for 10 min. Cells were rinsed with phosphate buffered saline (PBS) and incubated with 0.1% triton X-100 for 30 min. After three 5-min washings, cells were blocked with 5% BSA, then incubated for 2 h with phallacidin (10 U/ml) or α-, β-tubulin antibodies (1:200 dilution) for labeling F-actin or microtubules, respectively, followed by wash. Secondary antibodies, fluorescein goat anti-mouse for α-, β-tubulin were applied for 1 h. Nuclei were stained with DAPI (3 μmol/l) for 30-

min. After rinsing with PBS, slides were coverslipped. Mitochondria, F-actin or microtubules were detected under fluorescence microscopy with excitation/emission wavelength of 579/599 nm for Mito Tracker Red, and 492/520 nm for phallacidin or fluorescein, and 358/461 nm for DAPI labeling. The yellow fluorescence produced by overlapping red and green fluorescence identified either co-localization or superimposition of mitochondria with F-actin or microtubules. All fluorescence probes and antibodies were purchased from Molecular Probes.

2.3 Preparation of coronary arterioles

Human coronary arterioles (HCAs) were isolated from the right atrial appendage of subjects undergoing cardiac surgery as described previously [30]. HCAs were dissected from the endocardial surface of the appendage and prepared for histofluorescence and videomicroscopy. Protocols were approved by the Medical College of Wisconsin Institutional Review Board. The investigation conformed with the principles outlined in the Declaration of Helsinki.

2.4 Detection of F-actin and microtubules in human coronary arteriolar endothelial layer

HCAs with and without 20-min intraluminal pre-incubation with cytochalasin D or nocodazole were exposed to no flow or flow for 5 min (time at which FMD achieves a steady state value) followed by 5 min fixation with 3.5% paraformaldehyde. Vessels were then opened lengthwise and placed on glass slides exposing the luminal surface. After washing, HCAs were incubated with 0.5% triton X-100 and blocked with 1% BSA. Phallotoxin 586 (5 U/ml), or α-, β-tubulin antibodies (1:200 dilution) was applied to the vessel for detecting F-actin or microtubules, respectively. Endothelium was identified by co-incubation with antibodies to Von Willebrand Factor (vWF, 1:150 dilution). Vessel segments were exposed to antibodies overnight at 4 °C. After washing, secondary antibodies (Alexa fluor 488 goat anti-rabbit IgG for VWF and Alexa fluor 594 goat anti-mouse IgG for tubulin) were applied for 30 min followed by wash. The endothelial cytoskeleton was examined using confocal microscopy with excitation wavelength of 579 nm and emission of 599 nm for detecting F-actin and microtubules, 488 and 516 nm for detecting vWF.

2.5 Fluorescence detection of flow-induced hydrogen peroxide and superoxide generation in HCAs

Dichlorodihydrofluorescein (DCFH) [31,35] and hydroethidine (HE) [31] were used to evaluate vascular production of H_2O_2 and $O_2^{\bullet-}$, respectively, during flow. Four HCAs from the same atria were exposed to either no flow (pressurized at 60 mmHg, 0 gradient), or flow at a pressure gradient of 100 cm H_2O, in the absence or presence of intraluminal perfusion of cytochalasin D (5 lmol/l) or nocodazole (1 μmol/l). One vessel (not pressurized) treated with PEG-CAT (500 U/ml) and superoxide dismutase (150 U/ml), served as control for non-specific fluorescence. DCFH (5 μmol/l) and HE (5 μmol/l) were added in a light-protected chamber for 30 min at 37 °C either during flow or static conditions. Vessel segments were then washed with fresh PSS solution, and removed for fluorescence microscopy. DCFH and Oxy-ethidium (oxy-Etd, the fluorescent product of the reaction of HE with $O_2^{\bullet-}$) were excited at 488 and 585 nm, and emission was measured at 526 and 620 nm, respectively. Control and experimental tissues were examined in parallel and images recorded using the same computer-specified gain and intensity settings. Images were analyzed for intensity of fluorescence within a user-defined region of the arteriolar segment (maximal traceable area of the central portion of the vessel). Artifactual autofluorescent regions were manually eliminated from analysis. Relative average fluorescence intensity was normalized for surface area and compared between control and experimental vessels.

2.6 Detection of mitochondrial superoxide production in HCA endothelium

Mitochondrial $O_2^{\bullet-}$ generation in the presence or absence of flow was examined by MitoSox (5 μmol/l), a specific fluorescence probe for detecting mitochondrial $O_2^{\bullet-}$ production. MitoSox was perfused intraluminally for 15 min during flow. After washing, HCAs were coverslipped and examined with confocal microscopy at excitation/emission of 510/580 nm according to the instruction from the manufacturer (Molecular Probes).

2.7 Videomicroscopy

HCAs (100-150 lm) were mounted onto micropipettes for measurement of diameter [31]. Vessels were constricted by 30-50% by adding endothelin-1 (ET-1, 10^{-10} to 5×10^{-10} mol/l) when spontaneous myogenic tone was not sufficient to achieve the target reduction in diameter. Flow was produced by changing the heights of the cannulating reservoirs in equal and opposite directions to generate a pressure gradient of either 20 or 100 cm H_2O [36] in the absence or presence (20-min incubation) of inhibitors (cytochalasin D and nocodazole) or stabilizers (jasplakinolide, 100 nmol/l and taxol, 10 μmol/l) of cyto-skeleton elements. This pressure gradient is associated with steady-state flows of approximately 6 dyn/cm^2 and 34 dyn/cm^2, respectively [35]. After incubation, HCAs were re-constricted with endothelin-1 to a diameter similar to baseline diameter (without cytoskeleton inhibitors or stabilizers). A second flow-response curve was generated assessing percent dilation after each increment in flow. The responses to bradykinin (10^{-10} to 10^{-6} mol/l), an endothelium-dependent vasodilator and to papaverine (10^{-8} to 10^{-4} M), an endothelium-independent vasodilator that acts via a mechanism distinct from shear stress [2], were recorded in some vessels to assess the specificity of the effects of cytochalasin D and nocodazole.

2.8 Statistics

All data are expressed as mean ± SEM. The relative fluorescence intensities observed in arterioles exposed to static conditions or flow were compared using a two way ANOVA. Percent dilation was calculated as the percent change from preconstricted diameter to the diameter after flow or agonist (normalized to the maximal diameter measured after papaverine, 10^{-4} mol/l). Data from vessels exposed to flow before and after treatment with cytoskeleton antagonists were compared using a one-way ANOVA. All differences were judged to be significant at the level of $p < 0.05$.

3 Results

3.1 Characteristics of human coronary arterioles

Forty-eight HCAs with a mean maximal passive internal diameter of 138 ± 6 μm were used. Patient demographics including diagnoses are summarized in Table 1. Similar to previous reports [36], FMD was not altered by underlying diseases, including diabetes, hypertension, hypercholesterolemia, congestive heart failure, coronary artery disease, and myocardial infarction, or gender.

3.2 Localization and chemical disruption of cytoskeletal elements

We first examined the relative physical location of cyto-skeletal elements and mitochondria in human coronary endothelial cells. As shown in Fig. 1, there was substantial overlap between F-actin filaments and mitochondria in cultured human coronary endothelial cells (Fig. 1a). A similar relationship was observed between tubulin and mitochondria (Fig. 1b). The presence of F-actin and tubulin was further confirmed in HCA endothelium (Fig. 2a, g). Importantly cytochalaisin D and nocodazole treatment were effective in reducing the integrity of these cytoskeletal elements (Fig. 2d, j). Endothelial localization of cytoskeletal elements was confirmed by VWF staining (Fig. 2b, c, h, i). Intraluminal perfusion of cytochalasin D or

nocodazole had no discernable effect on the underlying smooth muscle cell cytoskeletal ultrastructure (data not shown).

3.3 Effect of disrupting the cytoskeleton on shear stress-induced superoxide and hydrogen peroxide generation

Since shear stress-induced $O_2^{\bullet-}$ and H_2O_2 generation are necessary for FMD, the role of cytoskeleton elements in shear stress-induced ROS generation was examined. The example shown in Fig. 3a shows cytochalasin D and nocodazole-sensitive shear stress-induced formation of $O_2^{\bullet-}$ and H_2O_2 in HCAs. Summary data (Fig. 3b) confirm increases in oxy-Etd and DCFH fluorescence intensities during flow (normalized to control, flow vs no flow, 1.71 ± 0.13 vs. 1.06 ± 0.09 and 1.67 ± 0.07 vs. 1.09 ± 0.12, respectively, $n = 5$, $p < 0.05$ vs. no flow) that were reduced by cytochalasin D (1.23 ± 0.05 and 1.31 ± 0.2) or nocodazole (1.11 ± 0.03 and 1.18 ± 0.1).

3.4 Effect of cytoskeletal disruption on shear stress-induced mitochondrial superoxide generation

Since the mitochondrial respiration chain is responsible for shear stress-induced ROS generation [31], we examined whether cytoskeleton elements were critical to the shear stress-induced generation of ROS from mitochondria. MitoSox (5 μmol/l), a fluorescence dye specifically localized within mitochondria was used (Fig. 4a). Fluorescence intensities (Fig. 4b) were increased in HCAs exposed to flow (3.50 ± 1.6-fold increase). This was reduced by cytochalasin D (0.51 ± 0.20, $n = 4$, $p < 0.05$ vs. flow) or nocodazole (0.81 ± 0.61, $n = 4$, $p < 0.05$ vs. flow) indicating that actin and microtubules are required for flow-mediated mitochondrial $O_2^{\bullet-}$ formation. Cytochalasin D induced slight reduction in fluorescence intensity in HCAs under no flow condition.

3.5 Role of cytoskeletal elements in flow-mediated dilation

As some vessels were preconstricted with ET-1, vasodilation to flow was first compared in HCAs preconstricted with myogenic tone (MT) or ET-1 to eliminate the possibility that ET-1 may influence the dilator response. A similar FMD was observed in vessels treated with or without ET-1 (% Max dilation, MT vs. ET-1, 20 cm H_2O: 19 ± 2% vs. 25 ± 3%; 100 cm H_2O: 69 ± 6% vs. 64 ± 6%, $n = 10$, p = NS).

Chemical inhibition of F-actin polymerization with cytochalasin D reduced FMD (% max dilation at 100 cm H_2O: 56 ± 3% vs. 26 ± 10%, $n = 6$, $p < 0.05$ vs. control; Fig. 5a). Depolymerization of microtubules with nocodazole had a similar effect (Fig. 5b, % max dilation at 100 cm H_2O: 58 ± 7% vs. 26 ± 11%, $n = 6$, $p < 0.05$ vs. control). Combined inhibition (Fig. 5c) produced an even greater reduction in FMD indicating the critical importance of these coexistant cytoskeletal elements in linking shear stress to mitochondrial-derived H_2O_2-mediated dilation. As shown in Fig. 6, neither cytochalasin D nor nocodazole affected the dilation to bradykinin (Fig. 6a, b) or papaverine (Fig. 6c, d) indicating specificity for shear stress-induced dilation.

3.6 Effect of stabilizing cytoskeletal elements on flow-mediated dilation in human coronary arterioles

Jasplakinolide (100 nmol/l), a stabilizer of actin filaments, and Taxol (10 μmol/l), an agent for promoting microtubular assembly and stability were used to examine the effect of enhancing cytoskeletal integrity on FMD. FMD was not altered by either jasplakinolide (% max dilation: 51 ± 6.3% vs. 63 ± 3%) or taxol (48 ± 7% vs. 52 ± 7%).

4 Discussion

The major new finding of this study is that an intact endothelial cytoskeleton is required for the shear stress-induced mitochondrial ROS formation and FMD. This is not unexpected since there is substantial overlap in function and anatomic organization of these components of the cellular cytoskeleton [17,33]. We also demonstrate a close physical relationship between cytoskeletal elements and mitochondria in human coronary endothelial cells. These findings suggest that in human coronary arterioles the endothelial cytoskeleton may be a key component of the signaling that links endothelial surface shear stress with mitochondrial release of H_2O_2, the EDHF that mediates flow-induced dilation.

4.1 Physiological importance of the endothelial cytoskeleton

The cytoskeleton of the cell is a complex mixture of cell structural proteins comprised of actin filaments, microtubules and intermediate filaments. The cytoskeleton anchors and arranges intracellular organelles, dissipates and transmits mechanical stresses across the cell, and serves as a scaffolding to promote interactions between signaling molecules. Numerous studies suggest that cytoskeletal integrity is necessary for a variety of cell-signaling responses, including opening of mitochondrial K_{ATP} channels [1], maintaining normal neuronal function [29], flow-induced dilation involving NOS activation [25], and cell growth [24]. The current study is the first to demonstrate that the cytoskeleton is necessary for mitochondrial mediated flow-induced vasodilation. The involvement of cytoskeletal elements in flow-mediated dilation is most likely located upstream of H_2O_2 generation based on our preliminary data that cytochalasin D or nocodazole had no effect on the dilation elicited by exogenous H_2O_2 (data not shown). In addition, we recently reported that bradykinin-induced dilation in HCAs is mediated by H_2O_2 [27]. However, this dilator response is not altered by inhibition of cytoskeletal elements (Fig. 6) indicating the pathway downstream of H_2O_2 remains intact. This observation is particularly relevant since it sheds light on a key regulatory mechanism of resistance vessel diameter in humans with coronary artery disease.

4.2 Endothelial cytoskeleton and shear stress-induced mitochondrial ROS generation and vasodilation

Shear forces on the luminal side of an artery can be converted into multiple cell signals including ion channel opening, membrane depolarization, activation of G-proteins, activation of integrins and focal adhesion kinases (FAK) [28,41], and stimulation of MAPK [41,42] and extracellular signal-regulated kinase (ERK) [42]. This diversity suggests a highly versatile endothelial mechanical signal transduction process. The endothelial cytoskeleton is ideally situated to contribute to this shear stress-induced signaling given its multitude of connections with the cell surface, integrins in the basement membrane, and intra-cellular organelles [17, 38,44,45]. For example in rat gracilis arterioles [43] and rabbit aortas [20], shear stress-induced NO-mediated dilation requires an intact cytoskeleton. The underlying mechanism by which cytoskeleton participates shear stress-induced NO generation involves activation of FAK and subsequent phosphorylation of paxillin and ERK [4,16]. Preincubation with cytochalasin D inhibits paxillin and ERK activation [37] as well as NO production [15]. These studies demonstrate an important role of cytoskeletal elements in regulating shear stress-induced NO formation and vasodilation. Interestingly the present paper shows that the same cellular structural elements are necessary for EDHF-mediated vasomotor responses that involved a separate signaling pathway that includes mitochondrial generation of ROS.

In this study, we show that the endothelial cytoskeleton plays a critical role in mediating shear stress-induced mitochondrial ROS production and subsequent dilation of HCAs. This conclusion is supported by the following evidence: (1) presence of a cytochalasin D- or nocodazoleinhibitable augmentation of endothelial $O_2^{\bullet-}$ and H_2O_2 production in response to

flow in HCAs; and (2) inhibition of FMD by disruption of endothelial cytoskeletal elements. The reduced FMD by cytoskeleton inhibitors is not a generalized effect and does not involve altered smooth muscle function. Rather it is specific to FMD and endothelial cells since dilations to the endothelium-dependent dilator bradykinin and to the endothelium-independent dilator papaverine are not altered by cytochalasin D or nocodazole. Endothelial localization was anticipated since cytoskeleton-modulating substances were delivered intraluminally where they would have preferential effects on the endothelium.

4.3 Study limitations

Because of the small size of HCAs isolated from the atrial appendage, we were not able to obtain primary endothelial cell cultures from human coronary arterioles. Therefore cultured human coronary endothelial cells from conduit arteries were used to examine anatomic linkages between the cytoskeleton and mitochondria. We cannot exclude the possibility that observations in conduit endothelial cells may differ from those in arteriolar vessels. However this concern is reduced by preliminary observations showing that shear stress-induced ROS production in HCAEC is also dependent upon mitochondrial respiration and intact cytoskeleton elements (data not shown), similar to that observed in HCA. Because of this concern, we limited use of HCAECs to anatomical localization experiments.

The fluorescence technique used to detect protein-protein interactions is widely used by others [22,23], however it is limited by poor spatial resolution. Thus it is not possible to determine the attachment sites of the cytoskeletal elements responsible for FMD. Future study using technique of fluorescence resonance energy transfer might provide more precise localization of intracellular communication between cytoskeletal elements and effector organelles which could include FAK, mitochondria, nucleus, or other cell organelles and structures.

It is tempting to speculate that a direct link between cytoskeleton and mitochondria mediate FMD, however from our data one can neither conclude that these connections exist nor that they are responsible for FMD. Stronger evidence would require determination of putative linkage proteins between cytoskeleton and mitochondria. In yeast, three mitochondrial outer membrane proteins, Mdm12p, Mdm10p and Mmm1p serve as cytoskeletal attachment sites [3,5,6]. Human homolog proteins have yet to be identified making it difficult to determine whether direct cytoskeletal-mitochondrial linkages might be responsible for signaling FMD. Cytoskeletal connection with multiple organelles and cellular structures provide numerous potential mechanisms for a role in FMD.

In this study, we observed that cytoskeletal disruption reduced not only flow-induced ROS production, but also decreased basal (no flow) ROS production (Fig. 4). All vessels were studied at a luminal pressure of 60 mmHg. It has been reported that vessel pressurization enhances cytochalasin D- or nocodazole-inhibitable polymerization of the cytoskeleton [10, 14]. Elevation of intraluminal pressure also increases ROS generation [39]. This might explain the basal increase in ROS generation that is reduced by cytoskeletal inhibition. The mechanism may differ from that involved in flow-mediated mitochondrial ROS generation, as was the focus of this study.

We examined only microtubules and filaments in this study and did not evaluate a role for intermediate filaments. The reason is twofold. First, microtubules have been shown in other studies to participate in the mechanism of FMD [43]. Second, intermediate filaments are a more heterogeneous set of structures and as a result there are no direct methods for disrupting them in HCA.

Surprisingly neither jasplakinolide nor taxol, cytoskeletal stabilizers, affected FMD. At least two possibilities exist for this observation. First, it is possible that flow evokes maximal

polymerization of the cytoskeleton and further enhancement by chemical stimulation cannot cause additive effect. Secondly, the dose of Jasplakinolide or taxol used may not have been sufficient to stimulate cytoskeletal polymerization. This possibility is unlikely, since the same concentration of jasplakinolide or taxol used by others significantly altered vasomotor tone in rabbit coronary [40] and cerebral [10] arteries. However, this observation does not alter our conclusion that cytoskeleton elements are required for FMD.

HCAs used in this study were from diseased patients. The results might not be applicable to normal human physiology. Nevertheless it is diseased subjects for whom treatments are directed and in whom a better understanding of the mechanisms of vasomotor responses are needed.

4.4 Summary

FMD occurs in coronary arterioles from patients with coronary disease through a unique mechanism involving mitochondrial production of ROS. The present study extends these observations by demonstrating a critical role for endothelial cytoskeletal elements in the signal transduction process linking cell surface shear with mitochondrial release of ROS.

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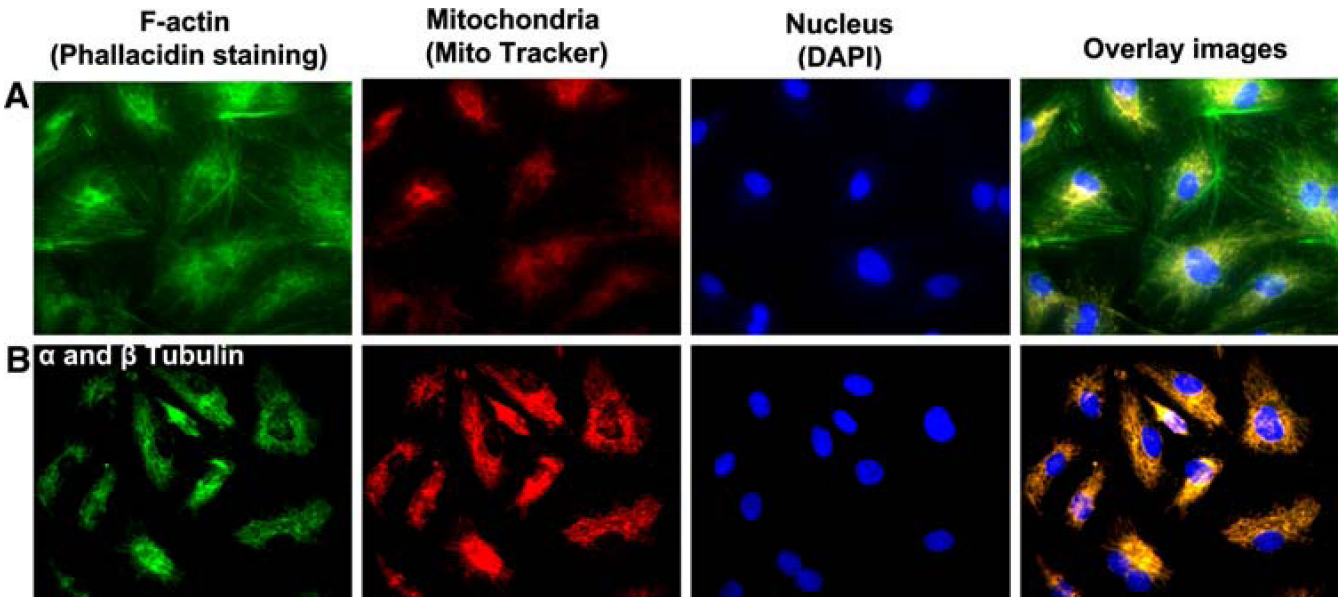

Fig. 1.

a Anatomical localization of F-actin (*green*), mitochondria (*red*), and nuclei (*blue*). F-actin filaments are closely associated with mitochondria as indicated in the overlay image. **b** Microtubules are detected by antibodies to α- and β-tubulin. Substantial overlapping fluorescence also exists between microtubules and mitochondria

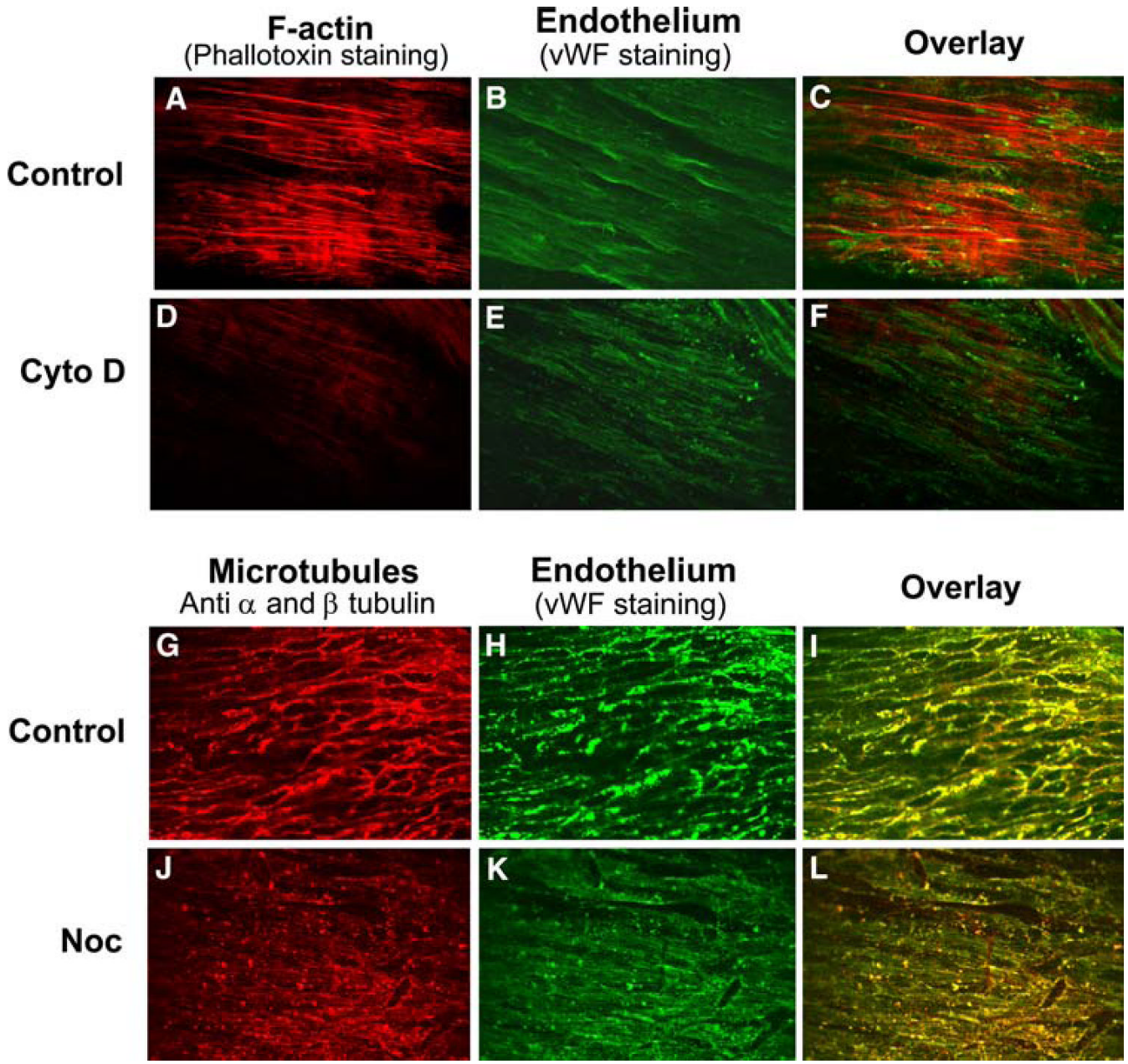

Fig. 2.

Detection of F-actin (**a**) and microtubules (**g**) in human coronary arteriolar endothelium. Cytochalasin D and nocodazole disrupted the integrity of F-actin (**d**) and microtubules (**j**). Von Willibrand Factor (*vWF*) staining (**b, e, h, k**) revealed in overlay images (**c, i**) that actin (**f**) and microtubular (**l**) disruption occurred in the endothelium

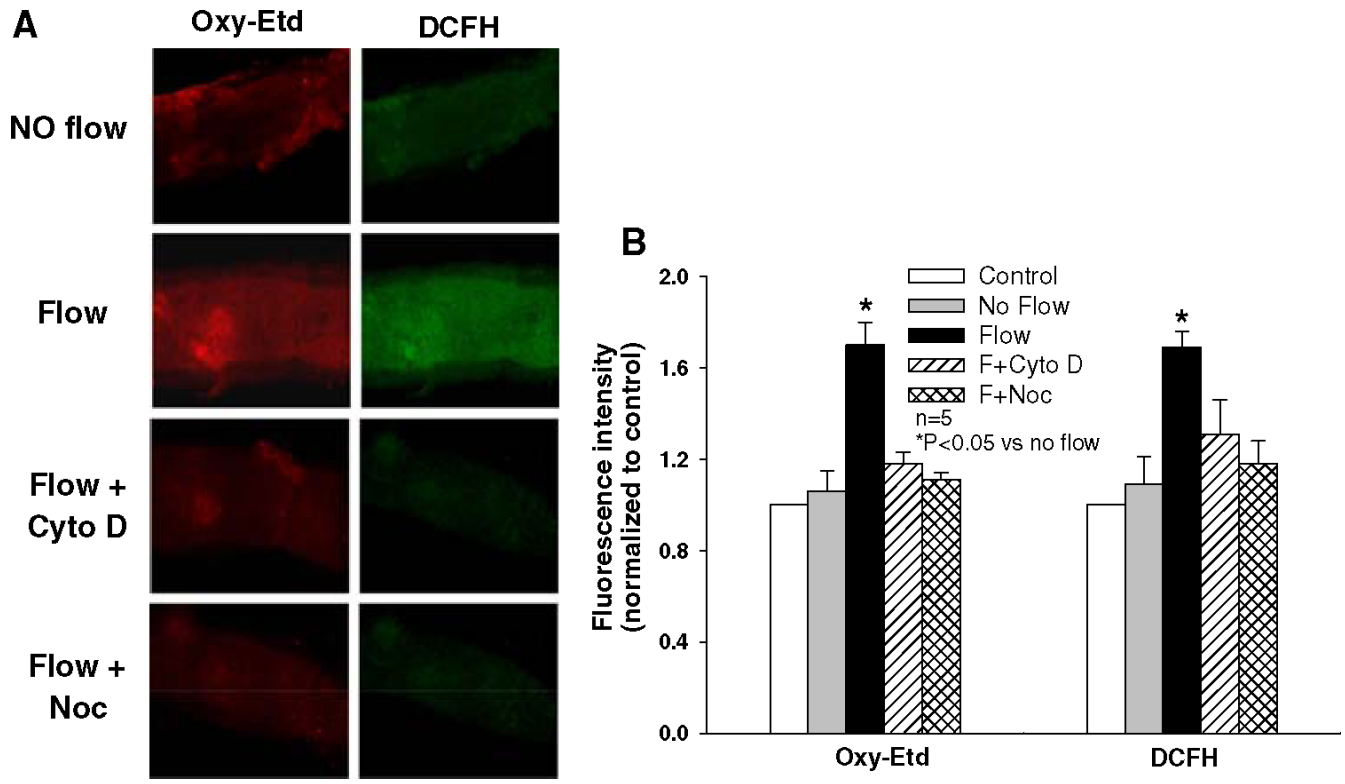

Fig. 3.

a HCAs were exposed to no flow, flow, or flow in the presence of cytochalasin D (*Cyto D*) or nocodazole (*Noc*). The enhanced *Oxy-Etd* and *DCFH* fluorescence intensities in HCAs by flow were reduced by *Cyto D* or *Noc*. **b** Summary of fluorescence intensities in five arteries for each group. Flowinduced increases in both Oxy-Etd and DCFH fluorescence intensities are blocked by Cyto D or Noc

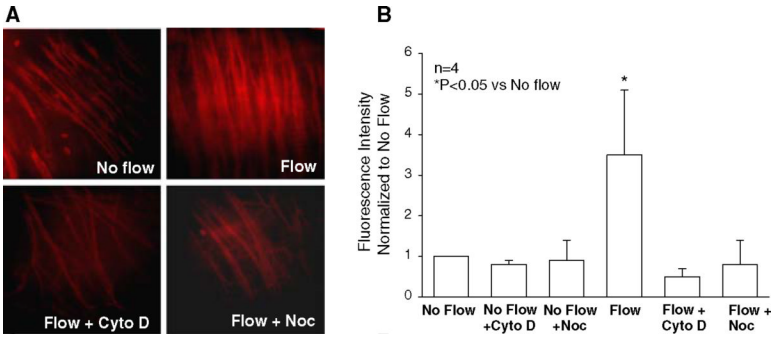

Fig. 4.

Confocal microscopic detection of flow-induced mitochondrial superoxide generation in HCA endothelium. **a** HCAs were exposed to no flow, no flow + Cyto D, no flow + Noc, flow, flow + Cyto D, or flow + Noc. Fluorescence intensities are markedly increased in HCA endothelium exposed flow and are decreased by Cyto D or Noc. b Summary of fluorescence intensities in each of the six conditions. The augmentation of fluorescence intensity induced by flow is blocked by disruption of F-actin or microtubules Cyto D slightly reduced fluorescence intensity under no flow condition

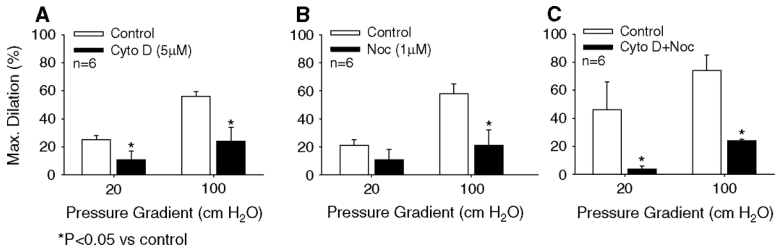

Fig. 5.

Effect of cytochalasin D (**a**), nocodazole (**b**) or the combination of both inhibitors (**c**) on flow-mediated dilation of HCAs. Dilation to increasing pressure gradient is attenuated by cytochalasin D or nocodazole alone and further reduced by the combination of inhibitors

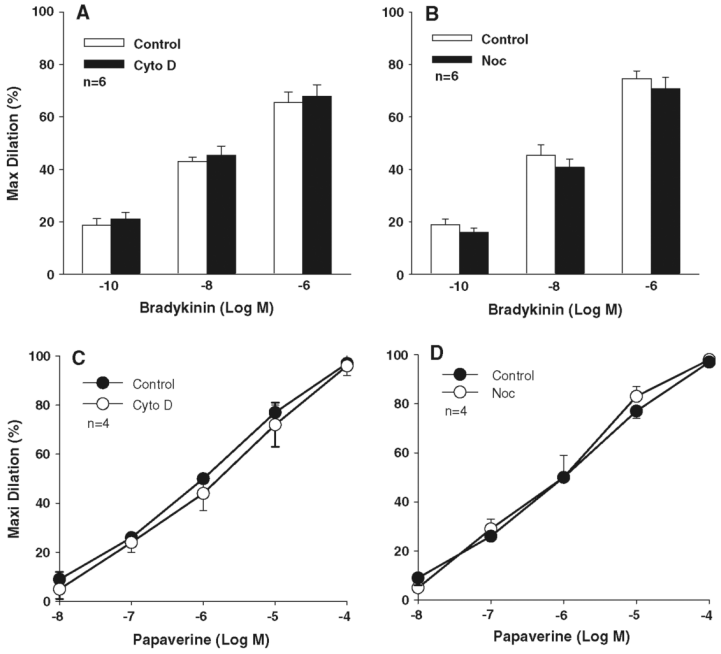

Fig. 6.

Effect of cytochalasin D or nocodazole on bradykinin-(**a, b**) and papaverine (**c, d**) induced dilation. Neither cytochalasin D nor nocodazole affected dilation to bradykinin or papaverine

Table 1

Demographics (n = 48)

	n
Sex (M/F)	29/19
Age (years)	68 ± 2
Diseases	
CAD	25
DM	11
HTN	22
HC	19
MI	7
CHF	6
Others	20

Data shown as mean ± SE, *n* indicates the no. of patients studied *M* male, *F* female, *CAD* coronary artery disease, *DM* diabetes mel-litus, *HTN* hypertension, *HC* hypercholesterolemia, *MI* myocardial infarction, *CHF* congestive heart failure