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Role of microtubules in attenuation of PepG-induced vascular endothelial dysfunction by atrial natriuretic peptide ☆

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Apart from control of circulating fluid, atrial natriuretic peptide (ANP) exhibits anti-inflammatory effects in the lung. However, molecular mechanisms of ANP anti-inflammatory effects are not well-understood. Peripheral microtubule (MT) dynamics is essential for agonist-induced regulation of vascular endothelial permeability. Here we studied the role of MT-dependent signaling in ANP protective effects against endothelial cell (EC) barrier dysfunction and acute lung injury induced by *Staphylococcus aureus*-derived peptidoglican-G (PepG). PepG-induced vascular endothelial dysfunction was accompanied by MT destabilization and disruption of MT network. ANP attenuated PepG-induced MT disassembly, NFκB signaling and activity of MT-associated Rho activator GEF-H1 leading to attenuation of EC inflammatory activation reflected by expression of adhesion molecules ICAM1 and VCAM1. ANP-induced EC barrier preservation and MT stabilization were linked to phosphorylation and inactivation of MT-depolymerizing protein stathmin. Expression of stathmin phosphorylation-deficient mutant abolished ANP protective effects against PepG-induced inflammation and EC permeability. In contrast, siRNA-mediated stathmin knockdown prevented PepG-induced peripheral MT disassembly and endothelial barrier dysfunction. ANP protective effects in a murine model of PepG-induced lung injury were associated with increased phosphorylation of stathmin, while exacerbated lung injury in the ANP knockout mice was accompanied by decreased pool of stable MT. Stathmin knockdown *in vivo* reversed exacerbation of lung injury in the ANP knockout mice. These results show a novel MT-mediated mechanism of endothelial barrier protection by ANP in pulmonary EC and animal model of PepG-induced lung injury via stathmin-dependent control of MT assembly.
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1. Introduction

Increased lung capillary leak and reduced alveolar liquid clearance capacity provoked by many pathologic factors including bacterial pathogens represent the major pathologic mechanisms of fluid accumulation in alveolar space. As a result, pulmonary edema combined with increased inflammatory cell infiltration in the lung leads to a life-threatening complication, the acute respiratory distress syndrome. Peptidoglycan (PepG) and lipoteichoic acid are two major cell wall components in gram-positive bacteria which stimulate inflammatory responses *in vivo* an *in vitro* via activation of toll-like receptors (TLRs) [1,2]. Of the ten TLRs known, only TLR2 has been clearly shown to be involved in the host defense against gram-positive bacteria [3,4]. Activation of TLR2 in endothelial cells leads to phosphorylation/activation of downstream targets including mitogen-activated protein kinases (MAPK) p42/p44, JNK1/2, and p38, nuclear factor kappa-B (NFκB) pathway [5]. Consistent with its key role in mediating inflammatory signaling from Gram-positive bacteria, siRNA-induced knockdown of TLR-2 decreased Raf phosphorylation and suppressed TLR2-mediated activation of Raf-MEK1/2-ERK1/2-IKK-NFκB cascade [6]. Increasing evidence suggests that, in addition to its role in body fluid control, atrial natriuretic peptide (ANP) exhibits direct anti-inflammatory and barrier effects on vascular endothelium which were demonstrated in the models of endothelial hyper-permeability induced by hypoxia, lysophospholipids and inflammatory mediators [7,8].

The two major ANP receptors, NPR-A and NPR-B act as membrane-associated guanylate cyclases [9], and elevation of cGMP levels is a primary response to ANP stimulation. ANP-induced elevation of cGMP decreased basal levels of lung EC permeability, attenuated pulmonary EC barrier dysfunction caused by hydrogen peroxide [10,11], and inhibited oxidant-induced pulmonary edema observed in perfused rabbit lungs [12]. However, ANP-mediated elevation of cGMP increased lung vascular permeability in the ischemia reperfusion model of lung injury [13],

Abbreviations: ANP, atrial natriuretic peptide; BAL, bronchoalveolar lavage fluid; cAMP, cyclic adenosine monophoshapte; cGMP, cyclic guanosine monophosphate; EC, endothelial cells; ECIS, electrical cell-substrate impedance sensing system; GEF, guanine nucleotide exchange factor; HPAEC, human pulmonary artery endothelial cells; MAPK, mitogen activated protein kinase; MT, microtubules; MPO, myeloperoxidase; MLC, myosin light chain; MYPT, myosin phosphatase targeting subunit1; nsRNA, non-specific RNA; PepG, peptidoglican-G; PKA, cAMP-dependent protein kinase; TER, transendothelial electrical resistance; TLR2, Toll-like receptor 2; XPerT, express permeability testing assay

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suggesting context-specific effects of cGMP and ANP in different models. Several reports also indicate the involvement of cAMP and cAMP-dependent protein kinase (PKA) in physiological responses elicited by ANP [14,15] including EC barrier protective effects mediated by Epac-Rap1-Rac1 signaling pathway [8]. The other report demonstrated PKA-independent activation of Rap1 by both cAMP and cGMP analogs and suggests activation of barrier protective Rap1 signaling through a cAMP/cGMP-regulated guanine nucleotide exchange factor [16]. ANP anti-inflammatory effects have been associated with attenuation of stress MAP kinase and NFκB cascade activities and Rho GTPase signaling [17,18], but precise molecular mechanisms of ANP-dependent attenuation of these pro-inflammatory pathways are not well-understood.

Regulation of vascular endothelial barrier is achieved via dynamic actin cytoskeletal remodeling in vascular endothelial cells (EC) coordinated with assembly and disassembly of cell-cell junctions [19]. Emerging evidence also indicates a critical role of crosstalk between actin networks and microtubules (MT) in precise regulation of EC permeability by chemical and mechanical factors [20,21]. MT-associated guanine nucleotide exchange factor H1 (GEF-H1) has been implicated in the MT-dependent regulation of Rho activity. In the MT-bound state, the nucleotide exchange activity of GEF-H1 is suppressed, whereas GEF-H1 release caused by MT disruption stimulates GEF-H1 [22].

MT dynamics controls many cellular processes including mitosis, locomotion, protein and organelle transport and permeability [23]. MT growth is regulated by a number of MT-associated proteins which control polymerization, depolymerization rates and MT stability. Stathmin is a regulator of MT dynamics which is expressed in endothelial cells and other cell types. In the unphosphorylated state, stathmin promotes MT destabilization by sequestration of soluble tubulin and by direct MT binding, which promotes MT shortening. Stathmin phosphorylation on one or more serine residues by PKA, Rac effector kinase PAK1 or other kinases reduces its MT-destabilizing activity [24]. This study elucidated the role of MT-dependent signaling in the EC barrier dysfunction and inflammatory activation induced by PepG *in vitro* and *in vivo*. We investigated the molecular mechanism of barrier-protective and anti-inflammatory effects of ANP via stathmin-mediated control of MT dynamics and MT-associated modulation of signaling pathways leading to EC permeability, inflammation and PepG-induced lung injury.

2. Materials and methods

2.1. Cell culture and reagents

Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza (East Rutherford, NJ) and used for experiments at passages 5-8. ANP and TRAP6 were purchased from Ana Spec (San Jose, CA). Reagents for immunofluorescence were purchased from Molecular Probes (Eugene, OR). HRP-linked anti-mouse and -rabbit IgG antibodies were obtained from Cell Signaling Inc. (Beverly, MA). Antibodies to phospho-myosin phosphatase targeting subunit1 (MYPT), GEF-H1, diphospho-myosin light chain (MLC), phospho-stathmin, and IκBα were from Cell Signaling Inc (Beverly, MA); stathmin and End-Binding protein-1 (EB1) were from BD Transduction Laboratories (San Diego, CA); ICAM-1 and VCAM-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Peptidoglycan G (PepG) polymer covers cell surface of *S. aureus* Gram positive bacteria. PepG of 99% purity isolated from *S. aureus*, cat-77140, was obtained from Fluka (Buchs, Switzerland). Unless specified, other biochemical reagents including β-Tubulin and acetylated tubulin antibodies were obtained from Sigma (St. Louis, MO).

2.2. siRNA and DNA transfections

To deplete endogenous stathmin, pre-designed human or mouse Stealth™ Select siRNA sets of standard purity were ordered from Invitrogen (Carlsbad, CA). Transfection of pulmonary EC culture with siRNA was performed as previously described [20]. Nonspecific, non-targeting siRNA (nsRNA) was used as a control treatment. After 72 h of transfection, cells were used for experiments or harvested for western blot verification of specific protein depletion. For *in vivo* experiments, we used polymer-based administration of non-specific or specific siRNA conjugated with polycation polyethilenimine PEI-22 as described in our previous studies [20,25]. Plasmid encoding stathmin-S63A mutant bearing a His-tag was provided by G. Bokoch (Scripps, La Jolla, CA) and was used for transient transfections of human pulmonary EC cultures according to protocols described elsewhere [20]. Control transfections were performed with empty vectors.

2.3. Cell imaging

Endothelial monolayers plated on glass cover slips were subjected to immunofluorescence staining with Texas Red phalloidin to visualize F-actin as previously described [20]. Quantitative analysis of paracellular gap formation in EC monolayers treated with ANP and PepG was performed as previously described [26].For microtubule quantification, cells were fixed with -20 °C methanol and immunostaining was carried out with β-tubulin or EB1 antibodies as described previously [27]. Briefly, after the cell boundaries were outlined, the concentric outline shapes reduced to 70% were applied to the images to mark peripheral (outer 30% of diameter) and central (inner 70% of diameter) regions. The integrated fluorescence density in the peripheral area was measured using MetaMorph software and was calculated as a percentage of the integrated fluorescence density in the total cell area. The results were normalized in each experiment. Similar methods were applied to EB1 quantification in fixed cells except that EB1 immunoreactivity was manually counted and results were not normalized. Minimum 10 cells per condition, in three experimental repeats were analyzed.

2.4. Measurement of EC permeability

The Express permeability testing assay (XPerT) was recently developed in our group [28] and now available from Millipore (Vascular Permeability Imaging Assay, cat. #17-10398). This assay is based on high affinity binding of avidin-conjugated FITC-labeled tracer to the biotinylated extracellular matrix proteins immobilized on the bottom of culture dishes. Permeability assays were performed in 96-well plates, and the fluorescence of matrix-bound FITC-avidin was measured on Victor X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). In permeability visualization experiments, cells were fixed with 3.7% formaldehyde. Images were acquired using Nikon video imaging system Eclipse TE 300 (Nikon, Tokyo, Japan) equipped with a digital camera (DKC 5000, Sony, Tokyo, Japan); 40× objective lenses were used. Images were processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA). Measurements of transendothelial electrical resistance (TER) across confluent HPAEC monolayers were performed using the electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy, NY) as previously described [29].

2.5. GEF-H1 activation assay

Plasmid encoding GST-tagged RhoA(G17A) mutant for bacterial expression was a generous gift from K. Szaszi (St. Michael's Hospital, Toronto, Canada). Active GEF-H1 was precipitated from cell lysates as previously described [30] using agarose beads with conjugated GST-tagged RhoA(G17A) mutant which cannot bind nucleotide and therefore has high affinity for activated GEFs [31]. Activated GEF-H1 in RhoA(G17A) pulldowns was detected by Western blotting and normalized to total GEF-H1 in cell lysates for each sample.

2.6. Microtubule reassembly assay and immunoblotting

Confluent HPAEC were stimulated with the agonist of interest for desired periods of time. MT disassembly was promoted by incubating of

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