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# The low molecular weight fraction of commercial human serum albumin induces acetylation of $\alpha$ -tubulin and reduces transcytosis in retinal endothelial cells



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## ABSTRACT

It has long been appreciated that the microtubule network plays a critical role in endothelial cell function. Chemical inhibition of tubulin polymerization has been shown to drastically increase endothelial permeability via interactions with the actin cytoskeleton. Conversely, stabilization of microtubules significantly decreases vascular permeability. The purpose of this investigation was to determine if the low molecular weight fraction of commercial 5% human serum albumin (LMWF5A) alters endothelial cell cytoskeletal dynamics and function. To investigate this, human retinal endothelial cells (HREC) were treated with LMWF5A and the acetylation of  $\alpha$ -tubulin was determined by immunofluorescent staining and immunoblotting. In addition, permeability assays were performed to evaluate functional changes. We found that HREC treated with LMWF5A exhibit a rapid increase in the amount and distribution of acetylated  $\alpha$ -tubulin. This was accompanied by a reduction in macromolecular permeability. Calcium depletion and inhibition of PI3-kinase reduced LMWF5A-induced acetylation while p38 MAPK inhibition potentiated this effect. These findings suggest that LMWF5A mediates changes in the microtubule network and reduces transcytosis in HREC.

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## 1. Introduction

Microtubules (MT) are filamentous cytoskeletal proteins that play a role in a number of essential cellular processes including division, migration, and intracellular transport [1]. To execute this diverse set of functions, MT continuously explore the cytoplasm

**Abbreviations:** LMWF5A, low molecular weight fraction of 5% albumin; HREC, human retinal endothelial cells; UTP, uridine-5'-triphosphate; EGTA, Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; HRP, streptavidin-horse-radish peroxidase.

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through a process termed dynamic instability [2]. Typically, MT are polymers composed of  $\alpha$  and  $\beta$  tubulin heterodimers arranged in an array of 13 head-to-tail protofilaments, aligned in parallel, to form hollow cylindrical tubes [3]. This configuration affords MT the ability to exist at variable lengths with GTP hydrolysis providing the trigger for stochastic phases of growth and collapse [2]. In order to polymerize, each tubulin heterodimer constitutively associates with a GTP molecule at each protein, with  $\beta$ -tubulin serving as a GTPase. As long as GTP-bound tubulin is added more rapidly than hydrolysis, a GTP-cap exists that stabilizes the molecule and growth continues. If the rate slows, however, the cap is lost and the polymer will rapidly depolymerize.

Due to these inherent chemical complexities, a host of conserved post translational modifications have evolved that help dictate function [4,5]. These decorations, in turn, regulate distinct interactions with a variety of microtubule-associated proteins

which can stabilize, sever, and/or exert conformational changes [2]. Unique among these modifications is the reversible acetylation of  $\alpha$ -tubulin at lysine-40 in that it resides on the luminal side, not on the outward surface or near the c-terminal tail [5]. On the basis of this observation it was believed that this modification was simply a gauge of the age or stability of the polymer but recent reports hint at more far-reaching and dynamic implications. Knock-out studies in mice have demonstrated that fibroblasts lacking  $\alpha$ Tat1/MEC-17, the major  $\alpha$ -tubulin acetyltransferase, exhibit reduced contact inhibition and cell adhesion [6]. Moreover, activation of HDAC6, a known tubulin deacetylase, by extracellular signal-related kinase has been shown to promote cellular migration [7]. These findings indicate that the functional consequences of tubulin acetylation are more encompassing than originally suspected.

It has long been appreciated that the MT are fundamental to endothelial cell function. For example, incubation of endothelial cells with paclitaxel, a microtubule stabilizing compound, significantly reduces permeability [8]. Conversely, destabilization of microtubules with nocodazole and vinblastine increases permeability through myosin light chain phosphorylation and Rho-GTPase activation [9]. Interestingly, the disruption of microtubules with 2-methoxyestradiol is attenuated by treatment with the p38 inhibitor, SB203580 [10]. As a whole, these findings indicate that an intimate relationship exists, across all components of the cytoskeleton, to regulate permeability. Also of note, a hallmark of the aforementioned investigations is an overall change in  $\alpha$ -tubulin acetylation status.

The purpose of this investigation was to explore the effect of the low molecular weight fraction of commercial 5% albumin (LMWF5A) on endothelial cytoskeletal dynamics and function. LMWF5A is a biologic derived from the less than 5 kDa fraction of human serum albumin currently under development as a therapeutic for osteoarthritis of the knee. In clinical trials, a single intra-articular injection of LMWF5A resulted in a significant 42.3% reduction in pain, which was observed 4 weeks following injection and persisted to the completion of the trial, versus saline controls [11]. *In vitro* experiments have also demonstrated that LMWF5A possess anti-inflammatory properties by inhibiting cytokine release from both stimulated peripheral blood mononuclear cells (PBMC) and T-cell lines [12–14]. Recent studies show that LMWF5A potentiates the release of an anti-inflammatory prostaglandin, (15d-PG)<sub>2</sub> from LPS-stimulated PBMC [14,15]. Furthermore, LMWF5A treatment of bone marrow-derived mesenchymal stem cells (BMMSC) reduced Rho GTPase activity and stress fiber formation [16]. Based on these observations, we hypothesized that LMWF5A reduces vascular permeability. To test this, we investigated *in vitro* permeability and monitored cytoskeletal changes by both cell imaging and immunoblotting. Our findings suggest that LMWF5A mediates changes to the microtubule network and reduces macromolecular permeability. These observations expand our knowledge of the mechanisms of LMWF5A and suggest that this biologic may alter microtubule dynamics and transcytosis.

## 2. Materials and methods

### 2.1. Reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. SB203580 was obtained from ThermoFisher Scientific (Waltham, MA). The  $\leq 5$  kDa filtrate of commercial 5% HSA was isolated by Ampio Pharmaceuticals (Englewood, CO) using tangential flow filtration (TFF) and a 5 kDa MWCO Hydrosart filter membrane (Sartorius Stedim Biotech GmbH, Germany).

### 2.2. Primary retinal endothelial cells

Primary human retinal endothelial cells (HREC) purchased from Cell Systems (Kirkland, WA) were cultured in EGM-2 growth medium supplemented as recommended (Lonza, Walkersville, MD) and used at passage 6 to 9.

### 2.3. Endothelial permeability assays

HREC were grown to confluence in 0.1  $\mu$ m pore transwell inserts (Thincerts; Greiner, Monroe NC) coated with 10  $\mu$ g/cm<sup>2</sup> fibronectin. Medium containing either saline, forskolin in saline (10  $\mu$ M final concentration), or LMWF5A mixed equally with EGM-2 medium was then added. To measure macromolecular permeability, streptavidin-horseradish peroxidase (HRP; ThermoFisher Scientific) was added to the upper chambers at a final concentration of 42 ng/ml. Colorimetric analysis was evaluated after 24 h by drawing 10  $\mu$ l from the bottom chamber and mixing with 100  $\mu$ l tetramethylbenzidine substrate solution (ThermoFisher Scientific). After 5 min, the reactions were stopped with 100  $\mu$ l 0.18 M H<sub>2</sub>SO<sub>4</sub> and the absorbance was measured at 450 nm (Spectra Max M<sub>5</sub> microplate reader; Molecular Devices, Sunnyvale, CA). Resistive changes were measured by growing HREC to confluence on fibronectin-coated, 8W10E + electrode arrays attached to an ECIS Ztheta system (Applied Biophysics, Troy NY) in EGM-2 medium. Solutions were then replaced with either saline or LMWF5A mixed equally with EGM-2, and impedance was monitored at 4000 Hz for 48 h, with data presented as normalized resistance.

### 2.4. Immunofluorescence staining

HREC were grown on glass bottom 24-well tissue culture plates (Cellvis, Mountain View, CA) coated with 2% gelatin in EGM-2. Medium was then exchanged with a combination of 500  $\mu$ l saline, working dilutions of compounds to be tested prepared in saline (final concentrations of 10  $\mu$ M LY294002, 10  $\mu$ M SB203580, 2 mM EGTA, 100  $\mu$ M UTP, and 0.2  $\mu$ M Thapsigargin), or LMWF5A together with 500  $\mu$ l EGM-2 and incubated for the indicated times. Following treatment, cells were fixed using 10% neutral buffered formalin for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min 4% goat serum (ThermoFisher Scientific) prepared in PBS was used to block the cells for 1 h then anti-acetylated  $\alpha$ -tubulin clone 6-11B-1 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) was added in blocking solution overnight at 4 °C. Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA) was then added for 1 h followed by DAPI counter staining (300 nM in PBS; ThermoFisher Scientific) for 5 min. Randomly selected frames were photographed on an inverted microscope (Zyla sCMOS camera; Andor, South Windsor, CT and eclipse Ti; Nikon, Melville, NY) and fluorescence intensity was measured using ImageJ software (<http://imagej.nih.gov/ij>) [17]. For normalization, the number of DAPI-stained nuclei was determined for each frame, and data are presented as median FU/DAPI objects.

### 2.5. Immunoblot analysis

HREC were grown to confluence on 2% gelatin coated 6-well culture dishes then treated as described for immunofluorescent staining with volumes scaled accordingly. Following treatment, cells were lysed in 100  $\mu$ l lysis buffer (Qproteome Mammalian Protein kit; Qiagen, Valencia, CA) according to manufacturer's instructions and cleared by centrifugation at 12,000  $\times$  g for 10 min at 4 °C. The lysates were separated by SDS-PAGE after boiling in Bolt Reducing Buffer and Bolt LDS Sample Buffer (ThermoFisher Scientific). Western blot analysis was performed using a mouse anti-

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