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The low molecular weight fraction of commercial human serum albumin induces acetylation of α-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 increases 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 α-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 α-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.

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 through a process termed dynamic instability [2]. Typically, MT are polymers composed of α and β 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 β-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

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, strepavidin-horseradish peroxidase.

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which can stabilize, sever, and/or exert conformational changes [2]. Unique among these modifications is the reversible acetylation of α-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 αTat1/MEC-17, the major α-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 then 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 α-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-PGJ_2) 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 ≤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 μm pore transwell inserts (Thincerts; Greiner, Monroe NC) coated with 10 $\mu g/cm^2$ fibronectin. Medium containing either saline, forskolin in saline (10 μM final concentration), or LMWF5A mixed equally with EGM-2 medium was then added. To measure macromolecular permeability, strepavidin-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 μl from the bottom chamber and mixing with 100 μl tetramethylbenzidine substrate solution (ThermoFisher Scientific). After 5 min, the reactions were stopped with 100 μl 0.18 M H_2SO_4 and the absorbance was measured at 450 nm (Spectra Max M_2^e 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 μl saline, working dilutions of compounds to be tested prepared in saline (final concentrations of 10 μM LY294002, 10 μM SB203580, 2 mM EGTA, 100 μM UTP, and 0.2 μM Thapsigargin), or LMWF5A together with 500 μl 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 α-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 μl lysis buffer (Qproteome Mammalian Protein kit; Qiagen, Valencia, CA) according to manufacturer's instructions and cleared by centrifugation at 12,000 × 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-

acetylated α-tubulin clone 6-11B-1 and goat anti-actin antibody mix (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) followed by a chicken Alexa Fluor 594-conjugated anti-mouse IgG and chicken Alexa Fluor 488-conjugated anti-goat IgG antibody mix (1:1000; Invitrogen, Carlsbad, CA). Immunoblots were visualized on a Kodak Image station (Carestream Health, Rochester, NY) with appropriate filter sets.

2.6. Data analysis

One-way ANOVA tests were performed with post hoc Bonferroni correction, and 95% confidence intervals were constructed using Excel with significance set at 0.05 (Microsoft; Redmond, WA).

3. Results

3.1. LMWF5A induces temporal and phenotypic changes in HREC α-tubulin acetylation

Previous studies demonstrated that LMWF5A treatment of BMMSC results in a reduction of cytoplasmic stress-fibers concurrent with the development of filopodia-like projections around the periphery of the cell [16]. In HREC, however, no appreciable change in f-actin was observed following treatment (data not shown). Instead, immunofluorescence (IF) staining revealed that 3 h after exposure to LMWF5A, HREC exhibited a marked increase in α-tubulin acetylation, a perceived marker of microtubule stabilization (Fig. 1A). Fig. 1B depicts a representative IF experiment in which temporal changes in LMWF5A-induced tubulin acetylation were tracked. A significant increase ($p < 0.01$; $n = 6$) over the saline control was observed at all time points tested: 1.5-fold at 30 min, 1.8-fold at 3 h, 1.7-fold at 6 h, and 1.3-fold at 24 h. These findings were confirmed by western blot analysis (Fig. 1C). In addition, this technique afforded the sensitivity to detect increased acetylation, manifesting after 10 min.

IF also showed that LMWF5A altered the distribution of acetylated tubulin in HREC (Supplementary Fig. 1). When viewed at higher magnification, acetylated α-tubulin in saline controls was primarily located in microtubule-organizing centers around the nucleus. In contrast, LMWF5A-treated HREC exhibited elevated cytoplasmic and perinuclear staining.

3.2. LY294002 reduces while SB203580 potentiates LMWF5A-induced acetylation of α-tubulin

Both gene-annotation enrichment and protein kinase array analysis of BMMSC indicated that treatment with LMWF5A activates PI3kinase/AKT pathways [16]. Moreover, evidence suggests that disassembly of microtubules by p38 MAPK contributes to endothelial cell permeability [8,10]. Therefore, we hypothesized that these pathways may be involved in the ability of LMWF5A to acetylate tubulin. For confirmation, HREC were treated with LMWF5A in the presence of specific inhibitors for both PI3-kinase and p38 MAPK and IF was performed after 3 h. As seen in Fig. 2A, inhibition of PI3-kinase with 10 μM LY294002 reduced LMWF5A-induced acetylation ($p < 0.025$ vs LMWF5A + DMSO; $n = 6$). When percent inhibitions were calculated for four separate experiments performed in triplicate, it was found that LY294002 reduced LMWF5A-induced acetylation by 24% (95% CI 29-19). Conversely, inhibition of p38 MAPK with SB203580 dramatically increased α-tubulin acetylation versus both saline-DMSO controls ($p < 0.01$; $n = 6$) and LMWF5A-DMSO-treated cells ($p < 0.025$; $n = 6$) by 57% (95% CI 63-51) and 222% (95% CI 236-208) respectively. A similar pattern emerged when analyzed by western blot after a 3-h incubation (Fig. 2B).

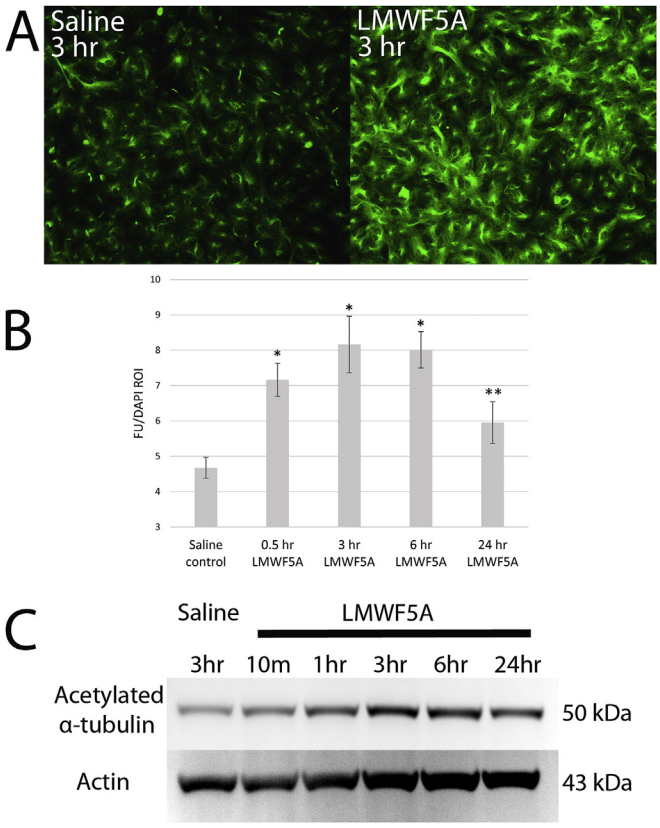

Fig. 1. LMWF5A induces acetylation of α-tubulin in HREC. **(A)** Immunofluorescence staining for acetylated α-tubulin in HREC treated with Saline or LMWF5A for 3 h. **(B)** Quantification of a representative immunofluorescence experiment. HREC were treated for 0.5, 3, 6, or 24 h and stained for acetylated α-tubulin. Data presented as mean FU normalized to the number of cells determined by DAPI counter staining ± SD (One-way ANOVA followed by Bonferroni's post hoc, * = $p < 0.01$ versus Saline, ** = $p < 0.02$ versus 3 h LWWF5A, $n = 6$). **(C)** Representative western blot performed for acetylated α-tubulin on lysates from HREC treated with LMWF5A for 0.5, 3, 6, or 24 h. Densitometry was then performed and normalized to actin loading.

3.3. Calcium and LMWF5A-induced acetylation of α-tubulin

In vitro polymerization assays using isolated α-tubulin demonstrate that calcium triggers disassembly of the polymer [18]. Taken together with the fact that PI3-kinase can activate second messengers that release calcium from internal stores, we sought to explore how calcium affects LMWF5A-induced α-tubulin acetylation. Fig. 3A provides a representative IF experiment in which HREC were exposed to LMWF5A in the presence of EGTA, uridine-5'triphosphate (UTP), or thapsigargin. Chelation of extracellular calcium with 2 mM EGTA reduced both the level of acetylated α-tubulin in saline controls ($p < 0.007$; $n = 3$) as well as attenuated LMWF5A-induced increases ($p < 0.02$; $n = 3$, Fig. 3A). Inhibition of sarco/endoplasmic reticulum calcium ATPase with thapsigargin, which causes calcium store depletion, also reduced LMWF5A-induced acetylation ($p < 0.02$; $n = 3$, Fig. 3A). In saline-treated controls, thapsigargin trended toward a significant reduction but did not survive post hoc correction. On the other hand, 100 μM UTP, an agonist of purinergic receptors that induces the release of calcium from intracellular stores, increased the detectable amount of α-tubulin acetylation following treatment with LMWF5A (Fig. 3A). Combined percent changes calculated for four separate experiments performed in triplicate showed that EGTA and thapsigargin reduced LMWF5A-induced α-tubulin acetylation by 100% (95% CI 138-62) and 49% (95% CI 56-43) respectively while UTP increased

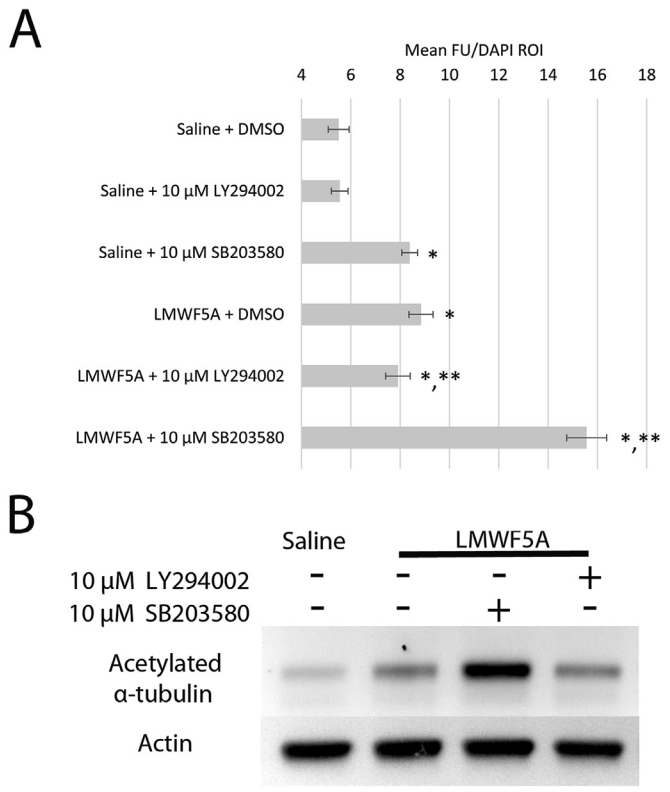

Fig. 2. Inhibition of PI3-kinase reduces while inhibition of p38 MAPK potentiates LMWF5A induced acetylation of α-tubulin. **(A)** Representative immunofluorescence of HREC exposed to LMWF5A for 3 h in the presence of specific inhibitors to PI3-kinase (10 μM LY294002) or p38 MAPK (10 μM SB203580). Data presented as mean FU normalized to number of cells determined by DAPI counter staining ± SD (One-way ANOVA followed by Bonferroni's post hoc, * = $p < 0.01$ versus Saline + DMSO, ** = $p < 0.025$ versus LWWF5A + DMSO, n = 6). **(B)** Representative western blot ofacetylated α-tubulin in lysates from HREC treated with LMWF5A for 3 h in the presence of specific inhibitors. Densitometry was normalized to actin loading.

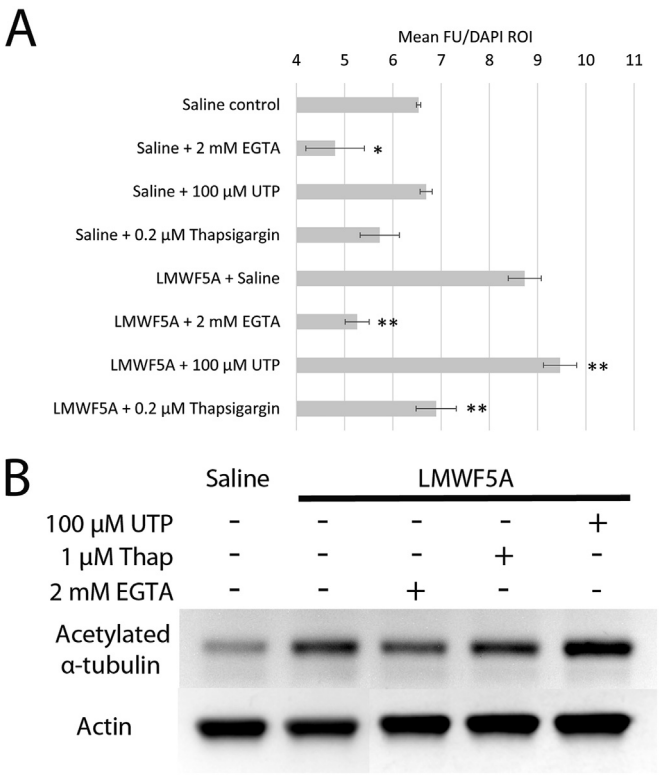

Fig. 3. Calcium dependence of LMWF-induced acetylation of α-tubulin. **(A)** Representative immunofluorescence of HREC exposed to LMWF5A for 3 h in the presence 2 mM EGTA, 100 μM UTP, or 0.2 μM Thapsigargin. Data presented as mean FU normalized to number of cells determined by DAPI counter staining ± SD (One-way ANOVA followed by Bonferroni's post hoc, * = $p < 0.0007$ versus Saline, ** = $p < 0.02$ versus LWWF5A + Saline, n = 3). **(B)** Representative western blot performed for acetylated α-tubulin in lysates from HREC treated with LMWF5A for 3 h in the presence of EGTA, UTP, or Thapsigargin. Densitometry was normalized to actin loading.

acetylation by 18% (95% CI 22-13). Protein lysates of HREC treated under these condition were also subjected to immunoblot analysis. Once again, when corrected for loading, EGTA and thapsigargin reduced and UTP enhanced acetylation of α-tubulin by LMWF5A (Fig. 3B). These findings reflect the importance of calcium and suggest that Ca^{2+} released from internal stores drives acetylation of α-tubulin following LMWF5A treatment.

3.4. Effect of LWMF5A on retinal endothelial cell permeability

To investigate the functional implications of LMWF5A treatment, *in vitro* permeability assays were employed. In the first, passage of HRP was determined across confluent monolayers of HREC established on porous transwell inserts. As seen in Fig. 4A, LMWF5A significantly reduced HRP permeability in this model by 48% as compared to saline-treated controls ($p < 0.025$; n = 3). A similar reduction was achieved by treatment with 10 μM forskolin.

Having established that LMWF5A decreases macromolecular permeability, trans-endothelial electrical resistance was then monitored for 48 h following treatment. In this assay, an immediate increase in resistance was observed after exposure to LMWF5A, lasting 30 min, with a subsequent reduction of 2–5% for approximately 15 h as compared to saline (Fig. 4B). After 24 h, however, LMWF5A-treated cells exhibited an increase in resistance that persisted to completion of the experiment. Taken together, these data suggest that LMWF5A treatment initially reduces transcytosis, then of offers protection against the breakdown of barrier function that corresponds with medium exhaustion.

4. Discussion

In the present investigation, we provide evidence that treatment of HREC with LMWF5A induces a rapid, calcium-dependent acetylation of α-tubulin. Mechanistically, it appears that LMWF5A-induced acetylation of α-tubulin could be inversely governed by PI3-kinase and p38 MAPK. In our model, pharmacologic inhibition of PI3-kinase reduced, while p38 inhibition synergistically enhanced acetylation following treatment. Compelling evidence supports the observation that these pathways work in opposing fashion to control microtubule dynamics. On one hand, PI3-kinase stabilization of microtubules at the leading edge of fibroblasts is essential to migration [19]. In the other, p38 inhibition counters TNFα-induced microtubule disruption in pulmonary artery endothelial cells [8]. Interestingly, PI3-kinase inhibition increases sensitivity of tumor cells to microtubule depolymerization with vincristine [20]. This also seems to apply to other cellular functions since blockade of PI3-kinase promotes VEGF-induced activation and apoptosis in endothelial cells [21]. Furthermore, PI3-kinase protects against ventilator-induced vascular permeability in mouse models by inhibiting p38 MAPK signaling [22]. As a result, we speculate that LMWF5A treatment of HREC leads to direct inhibition of Lys-40 specific deacetylases and/or down-regulation of p38 MAPK through PI3-kinase-mediated cascades.

Our data also suggests that LMWF5A-induced acetylation of α-

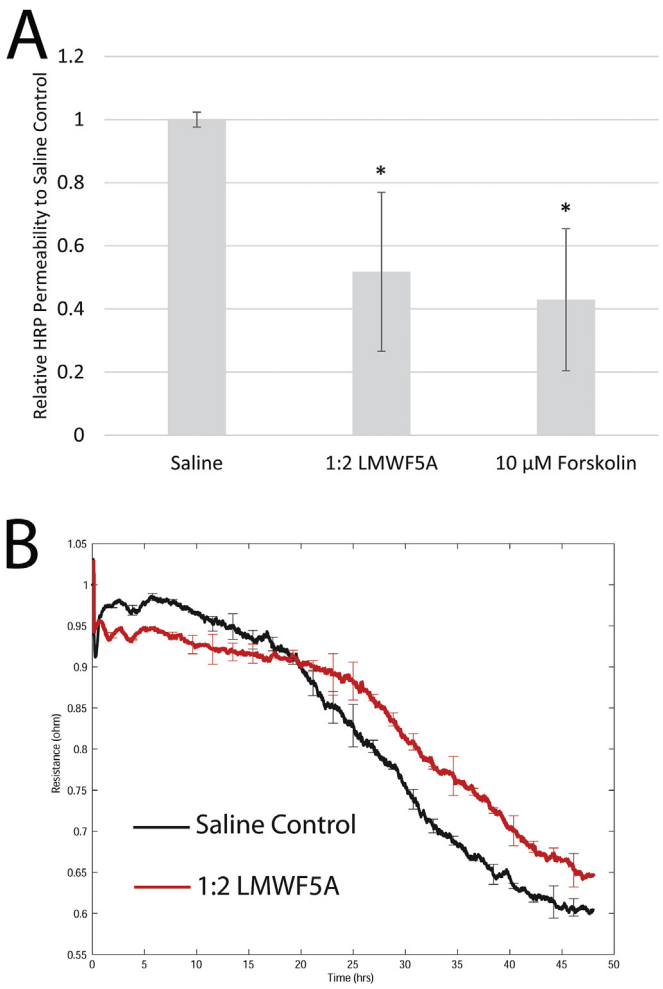

Fig. 4. LMWF5A reduces macromolecular permeability and late phase barrier disruption in HREC. **(A)** HREC were grown to confluence on transwell inserts then treated with Saline, LMWF5A, or 10 μM Forskolin. Macromolecular permeability over 24 h was measured by HRP, and relative permeability calculated versus saline controls. Data presented as Mean ± SD (One-way ANOVA followed by Bonferroni's post hoc, * = $p < 0.025$, n = 3) **(B)** HREC were grown to confluence on electrode arrays. Then trans-endothelial resistance was monitored for 48 h following treatment with Saline or LMWF5A.

tubulin is contingent on calcium. First we showed that calcium depletion, either externally by EGTA or from internal stores with thapsigargin, reduced acetylation. Moreover, activation of purinergic P2Y receptors using UTP provided an additive effect. Together, this implies that release of calcium from internal stores contributes to LMWF5A activity. New insight into the golgi apparatus and mitochondria may place this in biological context. It is now appreciated that the golgi stores calcium and serves as a non-centrosomal microtubule-organizing center [23,24]. Additionally, trafficking of mitochondria is regulated by kinesin-1 via the Ca^{2+}-binding protein, Miro [25]. Thus, localized calcium release may provide the means to rapidly nucleate and acetylate microtubules to power kinesin-driven motility. Does this explain the increased cytoplasmic acetylation of α-tubulin observed in LMWF5A treated HREC? Interestingly, a similar effect was observed by Lee et al. in which sodium-induced calcium influx increased tubulin acetylation by downregulating HDAC6 in medulloblastoma DAOY cells [26].

One of the primary functions of microtubules is to provide the scaffolding necessary for intracellular trafficking. Dynein and kinesin molecular motors track along these dynamic polymers as they explore the cytoplasm to deliver cargo [27]. It has also been well documented that the acetylation of tubulin controls both kinesin affinity and the directionality of transport [5,28]. Furthermore, transcytosis is a prominent mechanism for the transcellular passage of macromolecules across endothelial cells [29], thus, we propose that LMWF5A-induced microtubule acetylation reduces endocytosis and basolateral transcytosis by enhancing kinesin binding and activity. In support of this theory, we observed a slight decrease in resistance across HREC monolayers following treatment with LMWF5A. While counterintuitive, it has been demonstrated that inhibition of transcytosis in endothelial cells by blocking dynamin is accompanied by an increase in paracellular permeability [30]. Taken together with the fact that siRNA knockdown of dynein reduces the transendothelial transport of albumin [31], it seems likely that increased plus end trafficking, toward the periphery of the cell, contributes to LMWF5A activity.

In conclusion, we provide evidence that LMWF5A may reduce endothelial transcytosis by a microtubule mediated mechanism. Although further work is warranted to fully elucidate the pathways involved, our findings suggest that changes intracellular trafficking patterns could contribute to both the clinical and *in vitro* activities of LMWF5A.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.09.026.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.09.026.

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