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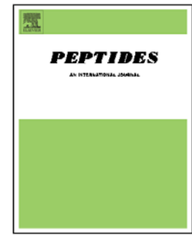

Adrenomedullin stabilizes the lymphatic endothelial barrier in vitro and in vivo

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ABSTRACT

The lymphatic vascular system functions to maintain fluid homeostasis by removing fluid from the interstitial space and returning it to venous circulation. This process is dependent upon the maintenance and modulation of a semi-permeable barrier between lymphatic endothelial cells of the lymphatic capillaries. However, our understanding of the lymphatic endothelial barrier and the molecular mechanisms that govern its function remains limited. Adrenomedullin (AM) is a 52 amino acid secreted peptide which has a wide range of effects on cardiovascular physiology and is required for the normal development of the lymphatic vascular system. Here, we report that AM can also modulate lymphatic permeability in cultured dermal microlymphatic endothelial cells (HMVEC-dLy). AM stimulation caused a reorganization of the tight junction protein ZO-1 and the adherens protein VE-cadherin at the plasma membrane, effectively tightening the endothelial barrier. Stabilization of the lymphatic endothelial barrier by AM occurred independently of changes in junctional protein gene expression and $AM^{-/-}$ endothelial cells showed no differences in the gene expression of junctional proteins compared to wildtype endothelial cells. Nevertheless, local administration of AM in the mouse tail decreased the rate of lymph uptake from the interstitial space into the lymphatic capillaries. Together, these data reveal a previously unrecognized role for AM in controlling lymphatic endothelial permeability and lymphatic flow through reorganization of junctional proteins.

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1. Introduction

The lymphatic vascular system is a blind-ended network of endothelial cell lined vessels that functions to maintain fluid homeostasis by unidirectionally transporting tissue fluid, extravasated plasma proteins, lipids, and cells from the interstitium to the circulatory system by way of the thoracic duct. When the lymphatic vascular system fails to function properly patients are at risk of developing serious and debilitating lymphedema. Pressure sensing, fibrillin-rich anchoring filaments that tether lymphatic endothelial cells (LECs) to the extracellular matrix contract in response to increases in interstitial pressure, thereby stretching LECs apart and facilitating lymph uptake into lymphatic capillaries. However, recent evidence suggests that LECs are also active participants in lymph transport through formation of an endothelial barrier that can regulate both ion and protein transport [3,7,19]. While these studies have implicated vascular endothelial growth factors A and C (VEGFA, VEGFC) and the intracellular signaling molecule cAMP as potential players in LEC permeability regulation, there remains a need to identify pharmacologically

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tractable targets that can efficiently modulate lymphatic permeability.

Adrenomedullin (AM) is a highly conserved 52 amino acid peptide vasodilator that is upregulated in a variety of cardiovascular conditions [12,13,23]. First recognized for its ability to maintain vascular smooth muscle tone, AM is also an important regulator of endothelial cell biology. For example, numerous in vitro studies have shown that AM is a potent angiogenic factor [10,20,32]. Our own recent studies using knockout mice have revealed that AM and its receptors are required for normal lymphatic vascular development [11].

With regard to endothelial permeability, AM treatment of human umbilical vein endothelial cell (HUVEC) monolayers dose dependently reduced hyperpermeability caused by inflammatory mediators including H_2O_2, thrombin, *E. coli* hemolysin and S. *aureus* α-toxin [2,14,16] The protective effect of AM on the endothelial barrier has also been shown in vivo in rat ileum exposed to S. *aureus* α-toxin and ex vivo in rabbit lungs exposed to H_2O_2 [2,14]. Further, in the blood-brain barrier, several studies have shown that AM treatment increased transendothelial electrical resistance thereby reducing endothelial permeability [18,21,22]. Taken together, these data suggest AM functions as a potent factor in maintaining the blood endothelial barrier; however, whether this function is conserved in LECs and to what extent remains unknown.

The purpose of this study was to explore the role of AM in the regulation of lymphatic permeability. We evaluated the effects of AM on the expression and localization of LEC junction components. Specifically, the response to AM treatment of the tight junction molecules Zonulus Occludin (ZO-1), Claudin-5, Claudin-12, and Junction Adhesion Molecular C (JAMC) as well as the endothelial-specific adherens protein VE-cadherin were assessed. Further, the ability of AM to modulate LEC permeability was measured utilizing both in vitro and in vivo approaches. The in vivo studies exploited the technique of fluorescent tail microlymphography and provided functional validation of our in vitro observations. Together, these data establish a previously unrecognized role of AM in stabilizing of the endothelial barrier and modulating lymphatic flow in vivo.

2. Materials and methods

2.1. Cell culture

Cryopreserved adult human dermal lymphatic microvascular endothelial cells (HMVEC-dLys) were obtained from Cambrex (Walkersville, MD) and maintained in Clonetics (East Rutherford, NJ) microvascular endothelial growth medium (EGM-2MV) and endothelial basal medium (EBM), respectively. Human AM and human $AM_{22–52}$ were purchased from American Peptide (Sunnyvale, CA), Forskolin from Sigma–Aldrich, and recombinant human VEGFA from Pierce Biotechnology. Goat anti-VE-cadherin (C-19) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-ZO-1 antibody (33-9100) was obtained from Zymed (San Francisco, CA). Cy2 and Cy3-labeled secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

2.2. Immunofluorescence labeling and microscopy

For ZO-1 and VE-cadherin localization, cells were grown on 0.2% gelatin coated coverslips until they achieved confluence. The medium was changed to serum-free conditions and the cells were stimulated with AM (100 nM), VEGFA (10 ng/mL) or vehicle (0.1% BSA) for 30 min. The cells were then washed twice with PBS with calcium and then fixed/permeabilized in ice-cold ethanol for 30 min. Cells were blocked with 3% BSA in PBS for 1 h at RT and incubated with primary antibody in 3% BSA overnight at 4 °C and with appropriate secondary antibody for 1 h at RT. Nuclei were labeled with Hoechst 33258 (Sigma). The coverslips were mounted on glass slides and images were obtained with a Nikon E800 microscope with a Hammamatsu ORCA-ER CCD camera using Metamorph Software (Molecular Devices) and processed in Adobe Photoshop 8.0 (Adobe).

2.3. Endothelial cell permeability

HMVEC-dLys permeability was studied as previously described [6,24,27]. Briefly, cells were plated at a density of 1×10^5 cells/cm^2 on gelatin-coated membranes (Corning Costar Transwells, 0.4 μm pore size, 6.5 mm diameter) and medium was changed every 24 h. Permeability of monolayers was measured in terms of Trypan Blue-BSA (TB-BSA) transfer [27]. At 96 h post seeding, membranes were incubated with test ligands in HBSS containing 0.03 M HEPES (HBSS/HEPES) in both the apical and basal-lateral chambers. In the apical chamber, 4% TB-BSA was added with or without test ligands followed by gentle shaking at 37 °C/5% CO_2 under sterile conditions for 90 min. At the end of the incubation, samples were taken from the lower chamber and the absorbance at 590 nm was measured. The relative permeability was calculated by dividing the OD of treated samples by vehicle control.

2.4. Gene expression analysis

Total RNA was isolated from cells using the Qiagen RNAEasy Mini Kit and DNase treated with Promega DNase per manufactures instructions. RNA was reverse transcribed using MMV reverse transcriptase (Invitrogen). cDNA was used for semi-quantitative RT-PCR or quantitative RT-PCR using gene-specific primers, SYBR Green (Stratagene), and the Stratagene MXP3000 and MxPro Software (Stratagene). The following murine primers sequences were used: VE-Cadherin: Forward: 5′-GGTGGCCAAAGACCCTGAC-3′ Reverse: 5′-ACTGGTCTTGCG-GATGGAGT-3′, JAMC: Forward: 5′-GCTGGGAGAGCACATG-CAA-3′ Reverse: 5′-CAGGAGCTCTGGGCTCACA-3′. Primers sequences for ZO-1, Claudin-5 and Claudin-12 were designed by the report by Holmes et al. [17]. The following human primers sequences were used: VE-Cadherin: Forward: 5′-GCCAGGTAT-GAGATCGTGGT-3′ Reverse: 5′-GTGTCTTCAGGCACGACAAA-3. Primer sets for human ZO-1, Claudin-5, Claudin-12, and JAMC were purchased from Qiagen.

2.5. Isolation of $AM^{-/-}$ endothelial cells

The generation and characterization of mice with targeted deletion of the *Adm* gene has been previously described [4]. To isolate $AM^{-/-}$ endothelial cells, timed matings were estab-

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