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RhoA S-nitrosylation as a regulatory mechanism influencing endothelial barrier function in response to G$^+$-bacterial toxins

F. Chen [a,b,*], Y. Wang [b], R. Rafikov [b], S. Haigh [b], W.B. Zhi [c], S. Kumar [b], P.T. Doulias [e], O. Rafikova [b], H. Pillich [f], T. Chakraborty [f], R. Lucas [b,d], A.D. Verin [b], J.D. Catravas [g], J.X. She [c], S.M. Black [b], D.J.R. Fulton [b,d,*]

[a] Department of Forensic Medicine, Nanjing Medical University, Nanjing, Jiangsu, China
[b] Vascular Biology Center, Augusta University, Augusta, Georgia 30912, USA
[c] Center for Biotechnology and Genomic Medicine, Augusta University, Augusta, Georgia 30912, USA
[d] Department of Pharmacology, Augusta University, Augusta, Georgia 30912, USA
[e] Department of Pediatrics, Children's Hospital of Philadelphia Research Institute, Philadelphia, PA 19104, USA
[f] Institute for Medical Microbiology, Justus Liebig University, Germany
[g] Old Dominion University, Norfolk, VA, USA

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ABSTRACT

Disruption of the endothelial barrier in response to Gram positive (G$^+$) bacterial toxins is a major complication of acute lung injury (ALI) and can be further aggravated by antibiotics which stimulate toxin release. The integrity of the pulmonary endothelial barrier is mediated by the balance of disruptive forces such as the small GTPase RhoA, and protective forces including endothelium-derived nitric oxide (NO). How NO protects against the barrier dysfunction is incompletely understood and our goal was to determine whether NO and S-nitrosylation can modulate RhoA activity and whether this mechanism is important for G$^+$ toxin-induced microvascular permeability. We found that the G$^+$ toxin listeriolysin-O (LLO) increased RhoA activity and that NO and S-NO donors inhibit RhoA activity. RhoA was robustly S-nitrosylated as determined by biotin-switch and mercury column analysis. MS revealed that three primary cysteine residues are S-nitrosylated including cys16, cys20 and cys159. Mutation of these residues to serine diminished S-nitrosylation to endogenous NO and mutant RhoA was less sensitive to inhibition by S-NO. G$^+$-toxins stimulated the denitrosylation of RhoA which was not mediated by S-nitrosoglutathione reductase (GSNOR), thioredoxin (TRX) or thiol-dependent enzyme activity but was instead stimulated directly by elevated calcium levels. Calcium-promoted the direct denitrosylation of WT but not mutant RhoA and mutant RhoA adenovirus was more effective than WT in disrupting the barrier integrity of human lung microvascular endothelial cells. In conclusion, we reveal a novel mechanism by which NO and S-nitrosylation reduces RhoA activity which may be of significance in the management of pulmonary endothelial permeability induced by G$^+$-toxins.

1. Introduction

Acute lung injury (or ALI) is a serious medical condition with a high mortality rate that can result in non-cardiogenic pulmonary edema and respiratory failure. A major cause of ALI is Gram positive (G$^+$) bacterial infections [1,2] with *Streptococcus pneumonia* accounting for up to 45% of all community-acquired pneumonia (CAP) cases in the US. In the United States alone, there are greater than 500,000 yearly cases of pneumonia and 40,000 pneumococcal-related deaths, which results in a health care burden exceeding 5 billion dollars [3]. Currently, there are no effective treatments that specifically target the pulmonary barrier dysfunction in ALI and a greater understanding of the cellular and molecular foundations of G$^+$-toxin induced pulmonary barrier dysfunction is needed.

The vascular endothelium is a monolayer of tightly connected endothelial cells that forms a barrier between the blood and the underlying interstitium which regulates the passage of fluid and cells. Dysfunction of the endothelial barrier arises from changes in cell behavior in response to signaling events that promote contraction, dissolution of adhesive complexes between cells and cell death. Many signaling pathways are involved including calcium,

* Corresponding authors at: Department of Forensic Medicine, Nanjing Medical University, Nanjing, Jiangsu, China; and Vascular Biology Center, Augusta University, 1459 Laney-Walker Blvd, Augusta, Georgia 30912, USA.
E-mail addresses: fchen@njmu.edu.cn (F. Chen), dfulton@augusta.edu (D.J.R. Fulton).

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MLC (myosin light chain)-dependent mechanisms [4], cytoskeletal rearrangements [5], disassembly of junctional proteins between cells [6], activation of PKC (protein kinase C) [7], alteration of nitric oxide (NO) signaling [8] and numerous others [9,10]. Unresolved disruption of the endothelial barrier results in the inappropriate loss of fluid from the vasculature and extensive pulmonary edema which are the major complications of acute respiratory distress syndrome (ARDS) and CAP [11].

The mortality rate of ALI remains unacceptably high despite a high level of support and the extensive use of antibiotics. A complicating factor in the use of bactericidal antibiotics is the release of toxins from G^+ bacteria, including LLO (listeriolysin-O, *Listeria monocytogenes*) and pneumolysin (PLY, *S. pneumoniae*). Both LLO and PLY belong to a family of cholesterol-dependent pore-forming cytolysins which form plasma membrane pores in the presence of calcium that stimulate the entry of calcium and other ions as well as stimulate intracellular signaling [7,8]. The mechanisms by which G^+ toxins promote endothelial permeability are not fully understood.

Nitric Oxide (NO) is an important regulator of vascular homeostasis and pathophysiology. In blood vessels, the primary source of NO is the enzyme endothelial nitric oxide synthase (eNOS), which is found almost exclusively in the endothelium, and has been shown to regulate endothelial permeability [12,13]. Previously we showed that G^+ toxins induce eNOS uncoupling which results in increased superoxide production. This occurs in part through an increase in PKCα-dependent eNOS T495 phosphorylation which alters enzymatic fidelity to reduce NO and increase superoxide synthesis [8]. However, the particular role of NO in regulating endothelial permeability is poorly understood and complicated by the amount of NO, the vascular bed and the agonist [14]. NO signaling is primarily mediated by soluble guanylate cyclase and cGMP-dependent signaling in smooth muscle. In endothelial cells, there are higher concentrations of NO and lower levels of soluble guanylate cyclase and NO-dependent signaling can be mediated via an alternative pathway. Sulfhydryl groups on substrate proteins can be covalently modified by NO and its metabolites in a process called S-nitrosylation that can modulate the function of numerous substrate proteins [12,15–18]. S-nitrosylation is a reversible modification and the removal of S-NO is primarily mediated by two enzymes, the GSNOR (S-nitrosoglutathione reductase) and thioredoxin 1(Trx1) [19].

RhoA belongs to the family of small GTPases which are activated by the binding of GTP versus GDP [20]. RhoA is a key regulator of numerous cellular responses involved in barrier function including cell adhesion and contraction. Upon activation, RhoA translocates to the plasma membrane where it contributes to the loss of barrier function by destabilizing endothelial junction proteins and increasing myosin light chain phosphorylation to promote cell contraction [21]. The actions of RhoA are opposed by another GTPase, Rac1 and the loss of pulmonary barrier function in ALI is partly due to a shift in the balance of RhoA and Rac1 signaling where RhoA becomes dominant [22,23]. Several post-translational modifications have been shown to modulate the activity of Rho family proteins, including transglutamination [24], deamidation [25], glycosylation [26], ADP-ribosylation [26], adenylylation [27], and phosphorylation [27]. Our group has previously demonstrated the nitration of RhoA in response to LPS which induces enhanced activity through the changes in nucleotide cycling [23] but the role of other nitrosative modifications are not well understood.

However, the ability of NO (as opposed to ONOO-) to regulate the activity of RhoA in human lung microvascular endothelial cells (HLMVECs), i.e. whether NO directly S-nitrosylates RhoA and whether this pathway is important for G^+ toxin-mediated disrupted endothelial barrier function are not well described. Therefore, the goal of the current study was to determine the role of S-nitrosylation in the LLO-induced activation of RhoA. We found that RhoA activity is decreased by NO, that RhoA is an S-nitrosylated protein and identify a novel calcium-dependent mechanism by which G^+-toxins stimulate the denitrosylation of RhoA. We also identify three sites of S-nitrosylation on RhoA (C16, C20 and C159) and show that mutation of these cysteine residues to serine provides resistance against the inhibitory effects of NO and results in a form of RhoA that is more effective at promoting endothelial barrier disruption compared to WT.

2. Materials and methods

2.1. Cell culture and transfection

COS-7 and HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS [28,29]. Human Lung Microvascular Endothelial Cells (HLMVECs) were isolated and grown in house as previously described [8,23] or purchased from Lonza and grown in Endothelial Growth Medium-2-Microvessel (EGM-2MV) containing the requisite growth factors and 5%FBS (Lonza, Allendale, NJ) and used below passage 8. Cells were grown in a 5% CO_2 incubator at 37 °C and used from passage 2–8. For transfection, COS-7 and HEK-293 cells were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. HEK 293 cells stably expressing eNOS were generated using the Flp-In System (Invitrogen, Grand Island, NY) [30].

2.2. DNA constructs, antibodies and reagents

Plasmid DNA coding RhoA have been described previously [23]. The C16, 20, 159S triple mutant RhoA was generated by Q5® Site-Directed Mutagenesis Kit (NEB, Ipswich, MA) and confirmed by DNA sequencing. The antibody against RhoA was purchased from Cell Signaling. The G^+-bacterial toxin, listeriolysin (LLO), was purified as described previously [7].

GSNOR/TRX genes were transiently silenced using siRNA. The siRNA targeting GSNOR (siRNA ID: s1071) and TRX (siRNA ID: s2) as well as validated non-targeting controls were purchased from Applied Biosystems (Carlsbad, California). Lipofectamine® RNAiMAX transfection reagent was used to deliver the siRNA in HLMVECs (Invitrogen, Grand Island, NY).

2.3. Measurement of nitric oxide and cell viability

Confluent HLMVECs were treated with or without LLO in serum free medium for 1 h, and the accumulation of NO_2^- in the medium was measured by NO-specific chemiluminescence as previously described [8,31]. Cell viability was assessed by using the Cell Titer-Glo® Luminescent cell viability assay per the manufacturer's instructions (Promega, Madison, WI).

2.4. Measurement of RhoA activity

RhoA activity was measured using the RhoA G-LISA assay kit (Colorimetric format, Cat: # BK124) (Cytoskeleton, Denver, CO) following the manufacturer's instruction. In brief, approximately 1–2×10^6 HLMVECs were plated into 10 cm dishes and incubated overnight in EGM-2MV media. Following the indicated treatments, cells were lysed and the level of GTP-bound RhoA determined by the absorbance at 490 nm using a PolarSTAR luminometer (BMG Labtech, Cary, NC).