Lipopolysaccharide-induced Lung Injury Involves the Nitration-mediated Activation of RhoA*

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BACKGROUND: The activation of RhoA is a critical event in acute lung injury (ALI), but the role of nitrination in this process is unresolved.

RESULTS: The nitrination of RhoA at Tyr34 produced GEF-like conformational changes that stimulate RhoA by decreasing GDP binding.

CONCLUSION: We have identified a new mechanism of RhoA activation.

SIGNIFICANCE: Preventing RhoA nitrination may be useful for the management of ALI.

Acute lung injury (ALI) is characterized by increased endothelial hyperpermeability. Protein nitrination is involved in the endothelial barrier dysfunction in LPS-exposed mice. However, the nitrated proteins involved in this process have not been identified. The activation of the small GTPase RhoA is a critical event in the barrier disruption associated with LPS. Thus, in this study we evaluated the possible role of RhoA nitrination in this process. Mass spectroscopy identified a single nitration site, located at Tyr34 in RhoA. Tyr34 is located within the switch I region adjacent to the nucleotide-binding site. Utilizing this structure, we developed a peptide designated NipR1 (nitration inhibitory peptide for RhoA 1) to shield Tyr34 against nitrination. TAT-fused NipR1 attenuated RhoA nitrination and barrier disruption in LPS-challenged human lung microvascular endothelial cells. Further, treatment of mice with NipR1 attenuated vessel leakage and inflammatory cell infiltration and preserved lung function in a mouse model of ALI. Molecular dynamics simulations suggested that the mechanism by which Tyr34 nitrination stimulates RhoA activity was through a decrease in GDP binding to the protein caused by a conformational change within a region of Switch I, mimicking the conformational shift observed when RhoA is bound to a guanine nucleotide exchange factor. Stopped flow kinetic analysis was used to confirm this prediction. Thus, we have identified a new mechanism of nitrination-mediated RhoA activation involving in LPS-mediated endothelial barrier dysfunction and show the potential utility of “shielding” peptides to prevent RhoA nitrination in the management of ALI.

Acute lung injury (ALI)2 and acute respiratory distress syndrome (ARDS) represent the same disease spectrum defined by acute lung inflammation with increased vascular permeability. ALI/ARDS are both characterized by hypoxic respiratory insufficiency from noncardiogenic pulmonary edema. Patients with ALI have a PaO2/FiO2 of less than 300 mm Hg, whereas ARDS patients have a PaO2/FiO2 of less than 200 mm Hg (1). Despite significant investigation, therapeutic advancement for ALI/ARDS has been slow and insufficient. Treatment of ALI/ARDS patients with low volume mechanical ventilation is currently the only proven therapy (2), and mortality remains unacceptably high. A leading cause of ALI/ARDS is the excessive release of LPS from the outer membrane of Gram-negative bacteria (3). The subsequent disruption of the alveolar-capillary barrier leads to alveolar flooding and an accumulation of fluid and protein in the alveolar space, impairing gas exchange and precipitating respiratory distress. LPS exposure has been implicated in the increased overproduction of NO and superoxide, leading to the formation of peroxynitrite and protein nitrination (4, 5). Global up-regulation of cellular protein nitrination is implicated in the pathobiology of various diseases (6–9), and we have shown that pretreatment with a peroxynitrite scavenger decreases LPS-induced lung edema in mice, suggesting a key role for protein nitrination in the progression of ALI (5). Peroxynitrite is a powerful nitrating agent that can modify protein structure and function via the addition of a nitro group to the meta carbon of the aromatic ring of tyrosine (10). Nitrination of tyrosine shifts the pKa toward a more ionized state, introducing a negative charge on the modified tyrosine. This change in the local protein microenvironment can affect protein structure-function relationships, which can in turn alter the protein activity.

The small GTPases of the Rho protein family, RhoA, Rac1, and Cdc42, are key regulators of the actin cytoskeleton (11). Rac1 and RhoA have antagonistic effects on endothelial barrier function in the lung (12, 13). Rac1 is required for the assembly and maturation of endothelial junctions, whereas RhoA desta-
bilizes endothelial junctions by increasing isometric tension at the cell membrane (14) and subsequently increasing myosin driven contractility. Disruption of the balance between RhoA and Rac1 signaling can lead to several human pathologies including ALI/ARDS. It has been demonstrated that the activity of Rho family proteins can be modulated through post-translational modifications including deamidation (15) and transglutamination (16) that result in the activation of Rho proteins or ADP-ribosylation (17), glycosylation (17), adenyllylation (18), and phosphorylation (18) that result in inactivation. However, there is little information about the role of nitration on the activity the small GTPases during LPS challenge. Thus, in this study we investigated the role of protein nitration in the LPS-induced activation of RhoA. Our data elucidate a new mechanism by which the LPS-mediated increase in RhoA nitration leads to enhanced GTPase activity through alterations in nucleotide cycling. Further we have identified a single nitration site on RhoA (Tyr34) and show that a shielding peptide strategy based on this sequence attenuated LPS-mediated vascular barrier dysfunction both in culture and in the mouse lung.

MATERIALS AND METHODS

Isolation of Human Lung Microvascular Endothelial Cells—Isolation and culture of human lung microvascular endothelial cells (HLMVEC) were performed as described previously (19).

Western Blot Analysis—HLMVEC were lysed and centrifuged at 6000 × g, and the supernatant was collected as previously described (20, 21). Cell extracts were separated on 4–20% gels, electrophotoretherically transferred to PVDF membrane (Bio-Rad), and then blocked with 5% nonfat dry milk in Tris-buffered saline. The membranes were probed with antibodies to RhoA (Cell Signaling), eNOS (BD Biosciences), or 3-nitrotyrosine (Calbiochem). Reactive bands were visualized using chemiluminescence (Pierce) on a Kodak 440CF image station. Band intensity was quantified using Kodak one-dimensional image processing software. Protein loading was normalized by reprobing with mouse anti-α-actin.

Immunoprecipitation Analysis—HLMVEC were homogenized in immunoprecipitation buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM MgCl2, 1 mM EDTA, 2% glycerol supplemented with protease inhibitors). To precipitate the bound protein, 10 μl of a protein A/G-agarose suspension (EMD/Calbiochem) was added for 2 h at 4 °C. The samples were then centrifuged at 14,000 rpm for 5 min, the supernatant was removed, and the beads were washed three times with immunoprecipitation buffer. Twenty μl of 2× Laemmli buffer was added, and the samples were boiled for 5 min and analyzed as described above. Immunoprecipitation efficiency was normalized by reprobing with RhoA.

Endothelial Monolayer Resistance Determinations—The electrical resistance of the endothelial cell monolayer was measured with the electrical cell impedance sensor technique. In this system, the cells are cultured on gold plated electrodes. The change in resistance across the monolayer is measured through an amplifier attached to the arrays. Each study was performed when the resistance reached a plateau. The data were normalized to the initial voltage and plotted as a normalized resistance.

Analysis of RhoA Activity—RhoA activity levels were measured using the Rhotekin Rho-binding domain pulldown assay. Briefly, 2 × 106 cells were seeded in 10-cm dishes and incubated overnight in DMEM with 10% FBS and 5% antibiotics. The cells were then pretreated with the peroxynitrite scavenger, MynTMPyp (25 μM), for 30 min. The cells were treated with LPS (1 EU/ml) for 4 h. The level of active RhoA pulled down by the assay was measured by Western blot analysis.

Immunofluorescence—HLMVEC were grown on gelatin-coated coverslips and exposed to LPS in the presence or absence of the NipR1 peptides. The cells were permeabilized and blocked for 1 h at 25 °C in 5% BSA blocking buffer. A primary antibody against ZO-1 (Invitrogen; 1:100 dilution) was applied. Plates were incubated overnight at 4 °C, and then a secondary antibody (AlexaFluor) was added for 2 h. The slides were examined with Zeiss immunofluorescence microscope Axio Observer D1. The gaps between the cells were calculated using Zeiss Axio Observer software.

RhoA Protein Purification—The Bl-21 strain of Escherichia coli was transformed with a polyHis-pET47b plasmid containing human RhoA and RhoA Y34F mutant sequences. Isopropyl-β-D-thiogalactopyranoside (1 mM) was added, and the cells were incubated for 18–20 h at 25 °C. Bacteria were then harvested by centrifugation, and the pellet was immediately lysed in 40 mM Tris-HCl, 5% glycerol, 1 mg/ml lysozyme, 100 mM NaCl, protease inhibitor mixture, ribonuclease A (Sigma), and deoxyribonuclease I (Sigma). The pellet was gently rocked for 30 min, sonicated, and subjected to ultracentrifugation. The supernatant was loaded onto a Hisprep FF 16/10 column using binding buffer (40 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 30 mM imidazole) at 0.1 ml/min flow. The column was washed with 40 mM Tris-HCl, 100 mM NaCl, 5% glycerol, 30 mM imidazole using a flow rate of 1.5 ml/min. Elution of the histidine-tagged protein was accomplished using elution buffer (40 mM Tris-HCl, 300 mM NaCl, 5% glycerol, 30 mM imidazole) at 1.0 ml/min flow. Collected fractions were loaded for size exclusion gel filtration on a HiLoad 26/60 Superdex 75 column using gel filtration buffer (60 mM Tris-HCl, 100 mM NaCl, 5% glycerol) at 0.2 ml/min flow. Fractions were collected and analyzed by Coomassie Blue staining and Western blot. All purification steps were performed at 4 °C, and purified protein was stored at −80 °C.

Rapid Kinetic Analysis of Nucleotide Binding to RhoA—RhoA was treated with authentic peroxynitrite (500 μM, 30 min) to induce tyrosine nitration in buffered solution. For kinetic analyses, 2 μM of either mant-GTP or -GDP was mixed with RhoA (0.1 μM) in a stopped flow instrument (SX-20; Applied Biophysics). Increases in fluorescent intensity mant–nucleotide binding to RhoA were then measured using excitation at 350 nm and a cutoff filter of 395 nm for 0.2–1 s. Acquired kinetic curves for three to five experiments were averaged and fitted using the Pro-kinetist software to determine observed binding constants.

Peptide Synthesis—Peptides containing 9 amino acids from amino acids 31–39 of RhoA fused with the cell permeable TAT sequence were synthesized by Peptide 2.0 Inc. (Chantilly, VA). NipR1 contains the natural RhoA sequence, and NpR1F has a Y34F mutation. A second set of peptides containing a biotin moiety at the NH2 terminus were also synthesized.
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NipR1 Peptide Binding to RhoA—To assess peptide binding to RhoA, we utilized biotinylated NipR1 and NipR1F. Purified RhoA (1 μg) was mixed with increasing concentrations of each peptide in the presence of Neutra-avidin-agarose beads (Pearce) for 1 h. The samples were then washed three times with PBS, and bound RhoA was eluted with 2x Laemmli buffer followed by boiling for 5 min. Samples were then separated on 4–20% gradient gels, and the levels of RhoA were determined using Western blot analysis and an anti-RhoA antibody. In a separate set of studies, a similar pulldown assay was carried out in which NipR1 was mixed with RhoA protein or recombinant GFP protein to determine the selectivity of peptide binding. The dissociation constant, K_d, for NipR1 was then calculated by fitting three independent RhoA binding kinetics data.

RhoA Shielding Properties of NipR1—Purified RhoA (1 μg) or GFP (1 μg) proteins were incubated with the peroxynitrite donor, SIN-1 (250 μM) in the presence or absence of the NipR1 peptide (5 μM), and samples were incubated for 1 h at 37 °C. Samples were then separated on 4–20% gradient gels, and the levels of protein nitration were monitored using Western blotting and an antibody raised against 3-nitrotyrosine. Protein loading was normalized using a duplicate blot stained with Coomassie Blue.

Peroxynitrite Scavenging Capacity of NipR1—This was determined using peroxynitrite-dependent DHR123 fluorescence. NipR1 (5 μM) was incubated with SIN-1 (250 μM) for 1 h at 37 °C. Peroxynitrite production was then monitored by measuring the fluorescence of rhodamine 123 using excitation at 485 nm and emission at 545 nm in a Fluoroskan Ascent microtiter fluorometer. Peroxynitrite scavenging capacity of NipR1 was assessed using a MALDI plate for further MS analysis.

MALDI-TOF-TOF Mass Spectrometry—All spectra were taken on an ABSciex 5800 MALDI-TOF-TOF mass spectrometer in positive reflector mode (10 kV) with a matrix of α-cyano-4-hydroxycinnamic acid (CHCA). Masses were calibrated to known peptide standards. 5-μl aliquots of the RhoA chymotrypsin/trypsin digest were cleaned on a C18 ZipTip (Millipore). Bound peptides were desalted and then eluted with 2.5 μl of acidic acetonitrile (75% CH3CN, 0.1% TFA). The eluant was mixed with 2.5 μl of freshly prepared CHCA stock solution (20 mg/ml CHCA), and 1.5-μl portions of this mixture were spotted onto a MALDI sample plate for air drying. Peptide coverage of 93% was achieved for the RhoA sequence, and only the COOH terminus peptide corresponding to amino acids 180–213 was not found in the MS. MS/MS of the 1700.5 m/z peak was also performed in positive reflector mode with collision-induced dissociation. MS and MS/MS spectra were analyzed using Protein Pilot 3.0, Mascot Distiller, and PEAKS software packages. To evaluate NipR1 peptide nitration, we mixed the NipR1 peptide (50 μg) with purified RhoA (5 μg) in 10× PBS buffer. The samples were then exposed or not to authentic peroxynitrite (500 μM) for 30 min. Each sample was then desalted using a C-18 ZipTip column and then mixed with CHCA matrix. Samples were then spotted directly onto a MALDI plate for further MS analysis.

Experimental Mouse Protocol—The Committee on Animal Research at Georgia Regents University approved all animal protocols and procedures. Stock solutions of E. coli LPS (0111: B4) were prepared in saline. Male C57Bl/6 mice (10 weeks of age; Harlan Laboratories) received vehicle (saline) or 1 mg/kg pep tide via an intraperitoneal injection 6 h before intratracheal installation of LPS (6.75 × 10^6 endotoxin units/g of body weight). Mice were examined 24 h after LPS treatment. At the end of the treatment, the lungs were flushed with 3 ml of ice-cold PBS (5 mm EDTA), excised, dipped in saline, and blotted dry. A portion of the lung was quickly snap-frozen in liquid nitrogen, crushed to powder in a prechilled mortar, and stored at −80 °C. The remaining lung tissue was stored at −80 °C.

Isolation of Bronchoalveolar Lavage Fluid (BALF)—BALF was obtained by instilling and withdrawing 1 ml of PBS via a tracheal cannula. Part of the solution was used to determine protein levels in the BALF. In addition, cells were pelleted at 2500 × g for 10 min and resuspended in water for 15 s to lyse red blood cells. Leukocytes were resuspended in 1 ml of PBS, and total cell count was determined using a hemocytometer.

Analysis of Lung Function—Mice were anesthetized with pentobarbital (90 mg/kg, intraperitoneally), tracheostomized with a metal 1.2-mm (internal diameter) cannula, and connected to a Flexi Vent (Scireq Inc) ventilator. Ventilation was initiated at a tidal volume of 10 ml/kg and a rate of 150/min. A TLC maneuver was performed, followed by 15 s later, by a sinusoidal 1 Hz oscillation. Subsequently, an 8-s forced oscillatory signal (0.5–19.6 Hz) was applied, the mechanical input impedance of the respiratory system was calculated, and a model containing a constant phase tissue compartment was fit to input impedance to evaluate tissue elastance. Dynamic pressure-volume maneuvers were performed by stepwise increasing the airway pressure to 30 cm H2O and then reversing the process.

Immunohistochemical Analysis of the Mouse Lung—Lungs were inflated with 10% formalin under 15 cm H2O pressure and immersed in the same solution before tissue processing into paraffin-embedded blocks, and 4-μm sections were then cut stained with hematoxylin and eosin. Histopathological assessment was conducted by two researchers who were masked to treatment group. Hematoxylin- and eosin-stained sections were scored for the presence of neutrophils within the alveolar walls. Neutrophils in the interstitial space, the existence of hyaline membranes, proteinaceous debris filling the airspaces, and alveolar septal thickening as described previously (22).

MPO Staining—Sections (5 μm) were cut from paraffin blocks and mounted on treated slides (Superfrost+). Slides were air-dried overnight, placed in a 60 °C oven for 30 min, deparaffinized in xylene, and run through graded alcohol to distilled water. Endogenous peroxidase was quenched with 0.3% H2O2 for 5 min, followed by two rinses with distilled water. Slides were pretreated with Target pH6, (Dako), rinsed in distilled water, incubated in Power Block (Biogenex), rinsed in distilled water, placed in PBS for 5 min, and incubated with primary anti-myeloperoxidase (Cappel, 1:2000 dilution) for 30 min at 25 °C. After two rinses in PBS, slides were incubated with a peroxidase-labeled polymer conjugated to goat anti-rabbit secondary IgG (Envision+, Dako) for 30 min and rinsed in PBS. Bound antibody was detected with the DAB+ substrate kit, (Dako). Hematoxylin was used as a counterstain. Myeloperoxidase (MPO)-stained slides were then evaluated by scoring (0–4) for the presence of neutrophils within the alveolar walls.
Cytokine and Chemokine Detection in the Bronchoalveolar Lavage Fluid—Analytes (pg/ml) were assessed with the MCYTOMAG-70K assay (EMD Millipore) as previously described (23).

Statistical Analysis—Statistical analysis was performed using GraphPad Prism version 4.01 (GraphPad). The mean ± S.D. or S.E. was calculated for all samples, and the significance was determined either by the unpaired \(t\) test (for two groups) and analysis of variance (for at least three groups). For the analysis of variance, Newman-Keuls post hoc testing was employed. A value of \(p < 0.05\) was considered significant.

RESULTS

Endothelial NOS Contributes to the LPS-mediated Barrier Dysfunction in HLMVEC.—To examine whether protein nitration by peroxynitrite is a contributing factor in endothelial barrier disruption in response to LPS, we measured transendothelial resistance (TER) in HLMVEC in the presence or absence of...
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A

| precursor mass | PTM | amino acids | sequence       |
|----------------|-----|-------------|----------------|
| 1655.83        | +NO₂ | 26-39       | SKDQFPEVV³⁴VPTVF |

B

[Graph showing mass spectrometry data]
the peroxynitrite scavenger, MnTmPyp. We found that MnTmPyp pretreatment significantly attenuated the barrier disruption associated with LPS treatment (Fig. 1A). To evaluate the role played by NOS in the barrier dysfunction, we exposed HLMVEC to LPS in the presence or absence of ethylthiourea (ETU) (a nonspecific NOS inhibitor) or 1400 W (to inhibit the target enzyme) for 4 h.

**FIGURE 2.** Identification of Tyr34 as the nitration site on RhoA. HLMVEC were exposed or not to LPS (1 EU/ml) for 4 h. RhoA protein was then immunoprecipitated, run on a 4–20% gradient gel, and stained with Coomassie Blue. The RhoA band was then subjected to in-gel chymotrypsin/trypsin digestion, and the resulting peptide fragments were subjected to MALDI-TOF MS analysis (four runs). Peptides coverage was 93%. A, a single nitrated peptide with sequence SKDQFPEVY*VPTVF was identified in LPS-treated cells corresponding to a peptide of 1700.5 m/z (green line). This nitrated peptide was not found in untreated cells (black line). Further MS/MS analysis of the 1700.5 m/z peptide was obtained in positive reflector mode (three runs). Resulted spectrum was fitted to the peptide sequence (SKDQFEVY(NO2)VPTVF) from a human RhoA modified at Tyr34 by nitro group. B, solid lines represent computed masses that were found in MS/MS spectra with mass error less than 0.3 ppm, and dotted lines indicate predicted masses that were not found in the MS/MS.

**FIGURE 3.** Development of a nitration shielding peptide for RhoA to prevent Tyr34 nitration. A, a biotinylated peptide centered on Tyr34 containing the sequence QFPEVYVPTVF was synthesized, conjugated to the HIV TAT sequence. This peptide was designated NipR1 (nitration inhibitory peptide 1), and designed to interact with the flap region of the Switch I domain in RhoA (left panel). A phenylalanine-substituted peptide (NipR1F) was also synthesized to serve as a control (right panel). Each peptide (100 ng/ml) was mixed with recombinant human RhoA (0.1 mg) and a biotin pulldown assay run to evaluate the ability of each peptide to bind to RhoA. B, a representative image is shown from three independent experiments and demonstrates that each peptide has a high affinity for RhoA. C, increasing concentrations of biotinylated NipR1 (0.3, 1, 1.5, 5, and 30 μM) were mixed with purified RhoA and GFP proteins to calculate the respective binding constants. NipR1 binding to RhoA exhibited a characteristic affinity curve with a calculated $K_a$ of 2.35 ± 0.84, whereas NipR1 binds to GFP in a nonspecific manner. RhoA and GFP proteins were incubated in the presence or absence of NipR1 (5 μM) and exposed to the peroxynitrite donor, SIN-1 (250 μM) for 1 h. D, Western blot analysis for protein nitration demonstrates that RhoA nitration is reduced in the presence of NipR1, whereas NipR1 did not reduce GFP nitration. E, peroxynitrite production from SIN-1 (250 μM), measured using DHR123 fluorescence, was not reduced in the presence of NipR1 (5 μM). F, mass spectrometry analysis indicates that the exposure of the NipR1 (50 μg) peptide (2655.4 Da, upper panel) to peroxynitrite (500 μM) produced a +45 Da mass shift (2700.7 Da, lower panel) that is related to NO2 group addition to tyrosine. We also observed tyrosine oxidation. G, HLMVEC were exposed to nonbiotinylated NipR1 and NipR1F peptides (100 ng/ml, 30 min), and then the cells were treated with LPS (1 EU/ml). The increase in total nitrated proteins induced by LPS was not attenuated by either peptide. H, and I, NipR1, but not NipR1F reduced the LPS-mediated increase in both RhoA nitration (H) and RhoA activation (I). J and K, the decrease in RhoA activity in NipR1-exposed cells also attenuated the LPS-mediated decrease in TER (J) and reduced the gap formation between cells (K). The data are means ± S.E.; n = 3. *p < 0.05 versus untreated; †, p < 0.05 versus LPS alone. IB, immunoblot; IP, immunoprecipitation; 3-NT, 3-nitrotyrosine.
FIGURE 3—continued

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F

G

H

I

J

K

FIGURE 3—continued
ETU, but not 1400 W, markedly attenuated the LPS-mediated barrier disruption in HLMVEC (Fig. 1B). The important role of eNOS was then confirmed using a siRNA approach. Reducing eNOS protein levels improved HLMVEC barrier function when challenged with LPS (Fig. 1C).

\textbf{LPS Increases RhoA Nitration through the Nitration of Tyr^{34}}

Peroxynitrite produced during LPS exposure can affect different proteins via the nitration of tyrosine residues. Nitration of tyrosines can affect protein structure and function. One of the key regulators of endothelial barrier disruption in response to LPS is the small GTPase RhoA. Thus, we next examined whether RhoA activity is modulated by nitration. Our data indicate that LPS significantly increased RhoA activity in HLMVEC, and this was attenuated by the peroxynitrite scavenger, MnTMPyp (Fig. 1D). Further, we found that LPS induces RhoA nitration (Fig. 1E), and this was attenuated by MnTMPyp. MS was used to identify peaks that differed from the predicted masses by +45 Da (equal to nitro group addition) in RhoA peptide fragments obtained from LPS-challenged HLMVEC. Only one nitrated peptide was identified: SKDQFPEV(Y(NO$_2$))VPTVF located within the region of amino acids 26–39 (Fig. 2A). MS/MS was also carried out to confirm the peptide sequence and the position of the nitro group as Tyr$^{34}$ (Fig. 2B).

\textbf{Protective Effect of NipR1 Peptide on RhoA Nitration, RhoA Activity, and HLMVEC Barrier Function—}We next investigated whether we could specifically target RhoA and prevent its...
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FIGURE 5. NipR1 attenuates lung injury in mice exposed to LPS. Mice received vehicle, NipR1, or NipR1F (intraperitoneal, 0.1 mg/kg) 6 h prior to intratracheal instillation of vehicle or LPS (2 mg/kg). After 24 h, mice were anesthetized, and BALF was collected. Measurements of lung mechanics were also carried out. A and B, total white blood cell counts as well as protein concentration were significantly increased in the BALF after LPS exposure, and these increases were attenuated by NipR1, but not by NipR1F. C, the LPS-mediated decrease in body weight is also attenuated by NipR1, but not by NipR1F. Lung sections were examined for signs of inflammation after hematoxylin and eosin staining. D, representative micrographs are shown. E, the inflammatory response induced by LPS is reduced by NipR1, but not by NipR1F. F–H, the increase in MPO staining (F and G) and MPO activity (H) induced by LPS is reduced by NipR1, but not by NipR1F. The analysis of dynamic pressure-volume relationships in the mouse lung also shows that NipR1, but not NipR1F, prevents the LPS-mediated disruption of lung airway mechanics. I, the data represent pressure-volume loops for four groups with two curves: one for inhalation and one for exhalation events. The data are means ± S.E.; n = 4–48. *, p < 0.05 from control; †, p < 0.05 from LPS alone; ‡, p < 0.05 from NipR1F.

nitrated at Tyr34. To accomplish this, we utilized a shielding peptide strategy. Docking experiments led to the identification of a peptide designated NipR1 (nitration inhibitory peptide for RhoA). Docking experiments indicated this peptide would bind to the flap region (Fig. 3A). A peptide in which Tyr34 was substituted with a phenylalanine residue (NipR1F) was also synthesized as a control. To confirm binding, we carried out a pulldown assay using biotinylated NipR1 and NipR1F peptides and were able to demonstrate a strong interaction between RhoA and both peptides (Fig. 3B). To calculate the binding constant of the peptides, we utilized different concentrations of biotinylated NipR1 along with recombinant RhoA (see Fig. 6C) in a pulldown assay. We found very low $K_d = 2.35 \mu M$ for NipR1 that reflects a strong affinity to RhoA (Fig. 3C). To further confirm the specificity of NipR1 binding, we utilized a similar pulldown assay in conjunction with recombinant GFP protein, which has similar molecular size to RhoA. Our data indicate that GFP binds to NipR1 in a nonspecific manner and only at much higher concentrations than those in which RhoA binding occurs (Fig. 3C). In addition, we evaluated the ability of NipR1 to specifically prevent RhoA nitration. To do this we used a low concentration of NipR1 (5 \mu M) that is sufficient to bind RhoA but insufficient to bind to GFP. We then exposed the RhoA-NipR1 or GFP-NipR1 mixtures to SIN-1 (250 \mu M). Our data indicate that under these conditions, NipR1 attenuates RhoA nitration but does not prevent the nitration of GFP (Fig. 3D). To confirm that this effect is not due to direct peroxynitrite scavenging, we also monitored peroxynitrite-mediated DHR123 oxidation with and without NipR1. Our data indicate that the SIN-1-mediated increase in DHR123 fluorescence was not significantly reduced by NipR1 (Fig. 3E). To confirm that when shielding RhoA, the NipR1 peptide was nitrated, a mixture of recombinant RhoA with NipR1 was exposed or not to authentic peroxynitrite and subjected to MS. Our MS data indicate that in the presence of peroxynitrite, NipR1 is nitrated as evidenced by the presence of a peptide with predicted mass that is increased by +45 Da (equal to nitro group addition; Fig. 3F). Interestingly, we also observed the addition of one and two oxygen atoms to the peptide, indicating that tyrosine oxidation is also occurring (Fig. 3F). We next investigated whether the NipR1 peptide could shield RhoA against nitration in LPS-exposed HLMVEC. To accomplish this, NipR1 and NipR1F peptides were fused with the HIV TAT sequence to increase peptide permeability. Further, to evaluate the potential of shielding RhoA, rather than scavenging peroxynitrite, we utilized a low concentration of peptide (300 nM). To confirm this we initially evaluated effects on the increase in total protein nitration induced by LPS in HLMVEC. Our data indicate that this increase was unaffected by either peptide (Fig. 3G). However, the LPS-mediated increases in RhoA nitration (Fig. 3H) and activation (Fig. 3I) were attenuated by NipR1, but not NipR1F. In addition, NipR1, but not NipR1F, attenuated the LPS-mediated disruption of the HLMVEC barrier as determined both by preservation of TER (Fig. 3J) and a reduction in cell-cell gaps (Fig. 3K).
NipR1 Peptide Attenuates RhoA Nitration and Activation in the Lungs of LPS-treated Mice—To explore the therapeutic potential of NipR1, we utilized a mouse model of ALI induced by the intratracheal distillation of LPS. Mice were administered with 0.1 mg/kg of NipR1 or NipR1F peptides, 6 h prior to LPS exposure. Again to evaluate the potential of shielding RhoA, rather than scavenging peroxynitrite, we utilized a concentration of peptide, 4 μM, that is close to its K_d for RhoA (2.35 μM; Fig. 3C) to reduce the likelihood that NipR1 will act as a nonspecific peroxynitrite scavenger. Twenty-four hours post-intratracheal LPS treatment, we found that, as in HLMVEC, LPS induced RhoA activation in the mouse lung (Fig. 4A). The increase in RhoA activity was prevented in the mice pretreated with NipR1, but not NipRF1 (Fig. 4A). The LPS-induced nitration of RhoA in the mouse lung was also attenuated by NipR1, but not NipRF1 (Fig. 4B). Total RhoA protein levels (Fig. 4C) and basal RhoA nitration levels (Fig. 4D) were unchanged.

NipR1 Peptide Preserves Lung Function in LPS-treated Mice—Mice were used to perform additional physiological, biochemical, and morphological studies to evaluate the efficacy of the NipR1 peptide in attenuating symptoms of ALI. Cell infiltration into the BALF was significantly increased in LPS-treated animals (Fig. 5A). NipR1, but not NipRF1, reduced this increase (Fig. 5A). A similar protective effect was observed when we measured protein concentration in the BALF (Fig. 5B). Further, NipR1, but not NipRF1, was able to prevent the weight loss (presumably dehydration) associated with LPS exposure (Fig. 5C). We also assessed histopathological changes in the lungs. LPS induces severe alveolar damage that includes the presence of large amount of neutrophils and red blood cells in the alveolar and interstitial space, formation of hyaline membranes, septal thickening, and debris accumulation in the alveoli (Fig. 5D). Again, NipR1, but not NipRF1, reduced these pathological changes (Fig. 5, D and E). NipR1, but not NipRF1, also attenuated the LPS-induced increase in MPO staining in the alveolar space (Fig. 5E). The reduction in lung elastance induced by LPS was also prevented by NipR1 but not NipRF1 (Fig. 5F), indicative of a preservation of lung mechanics. Finally, our data indicate that NipR1 attenuated the LPS-mediated induction of multiple pro-inflammatory cytokines and chemokines (Table 1).

Elucidation of the Mechanism of Nitration-mediated RhoA Activation—Comparison of RhoA GTPase in different crystal structures associated with different catalytic stages revealed that the Switch I region of RhoA undergoes a major conformational change during the catalytic cycle. We named this subregion of Switch I, which changes the conformation change during catalytic cycling, the “flap” (amino acids 28–40). The flap has an unstructured, flexible characteristic (Fig. 6A). Tyr34 is located in the middle of the flap, and molecular dynamics simulations predicted that nitration of Tyr34 would lead to the opening of the flap similar to that seen in the RhoA-GEF complex crystal structure (Fig. 6B). This was predicted to increase nucleotide cycling by decreasing the affinity of RhoA for GDP, leading to faster GDP release, thus increasing RhoA activity. To test the validity of the molecular dynamics data, we performed kinetic studies. Human recombinant RhoA purified from a bacterial expression system transformed with a His_6-tagged RhoA (Fig. 6C) was subjected to rapid flow kinetics to evaluate GDP and GTP binding using fluorescently labeled guanine nucleotides. The exposure of RhoA to peroxynitrite markedly reduced the GDP binding constant (Fig. 6D). However, nitration of RhoA had no effect on the GTP binding constant (Fig. 6E). When we expressed and purified a RhoA mutant protein (not shown) in which the tyrosine residue was replaced by phenylalanine, we found it to be unable to bind GTP, and it was catalytically inactive (Fig. 6F). In addition, when expressed in HLMVEC, the Y34F RhoA mutant did not modulate either the increase in RhoA activity (Fig. 6, G and H) or the barrier disruption associated with LPS exposure (Fig. 6I).

### DISCUSSION

It is well established that the endothelial barrier is regulated by cytoskeletal reorganization controlled by the small GTPases: RhoA, Rac1, and Cdc42 (11). RhoA activation is critical in the regulation of the endothelial barrier (24, 25). Our data add significantly to the knowledge regarding RhoA activation in ALI by elucidating a new mechanism of activation mediated via nitration at a single tyrosine residue (Tyr34). This is surprising, because most proteins reported to undergo tyrosine nitration are inhibited (26, 27). Our data are important because they add to the growing literature elucidating the interactions between

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**TABLE 1**

| Cytokines | Type I Receptor | Control (pg/ml) | LPS (pg/ml) | NipR1 (pg/ml) | NipR1 + LPS (pg/ml) |
|-----------|----------------|-----------------|--------------|---------------|---------------------|
| IFN-g | IL-2 | 2.65 ± 0.23 | 0.11 ± 0.07 | 1.90 ± 0.17 | 1.90 ± 0.17 |
| MIP-1α | IFN-γ | 3.98 ± 0.33 | 0.034 ± 0.054 | 3.19 ± 0.29 | 3.19 ± 0.29 |
| IL-6 | IL-8 | 5.75 ± 0.65 | 3.81 ± 0.3 | 1.01 ± 0.14 | 1.01 ± 0.14 |
| IL-10 | TNF-α | 46.4 ± 8.8 | 27.2 ± 2.9 | 69 ± 12 | 268.4 ± 19.1 |
| IL-12 | MCP-1 | 2.31 ± 0.47 | 29.6 ± 4.0 | 1.31 ± 0.53 | 1.31 ± 0.53 |
| IL-13 | CXC chemokine | 2.20 ± 0.68 | 78.0 ± 10.3 | 3.33 ± 0.51 | 3.33 ± 0.51 |
| IL-15 | GM-CSF | 1.85 ± 0.40 | 54.5 ± 5.4 | 2.60 ± 1.29 | 2.60 ± 1.29 |

**a)** Significantly different from the control group, b) Significantly different from the LPS group. (⁎, p < 0.05; ⁎⁎, p < 0.01; ⁎⁎⁎, p < 0.001)
RhoA signaling and cellular redox status. Both superoxide and nitrogen dioxide have been shown to accelerate GDP release from RhoA, Rac1, and Cdc42, by affecting the GXXXXGKS/T/C motif (28). From these data, it was concluded that cysteine 18 and phenylalanine 28 (RhoA sequence) mediate the redox sensitivity of the small GTPases. However, RhoA has also been shown to be inactivated by redox agents because of formation of an intermolecular disulfide bond (29). Adding to these studies, our data have elucidated the involvement of RhoA protein nitration in endothelial barrier disruption. However, it is important to acknowledge that in addition to peroxynitrite, other reactive oxygen species or reactive nitrogen species, such as superoxide, H2O2, or NO, are generated during the development of ALI. Thus, these other ROS and RNS may also be involved in the activation and/or inhibition of RhoA (30). In our study we did not evaluate their potential role. However, it should be noted that the exposure of purified recombinant RhoA to authentic peroxynitrite was sufficient to stimulate nucleotide cycling, while the Y34F mutant is catalytically inactive. Thus, although other species may be involved, we suggest that Tyr34 is a main target for redox modification.

Tyr34 is located in a flexible region of RhoA within Switch I domain (31), that we have christened the flap region. This domain is important for nucleotide and magnesium binding within catalytic cavity. Changes in the molecular conformations of the flap domain have been shown to occur during catalytic cycling (32–34). The catalytic cycling of RhoA consists of three major steps (Fig. 6): 1) GTP binds to RhoA, which represents the active conformation of the enzyme with the flap being in a closed conformation; 2) after phosphate bond cleavage of GTP, RhoA returns to an inactive conformation with GDP bound and with the flap partially open; and 3) RhoA releases GDP from the inactive conformation, usually by GEF protein assistance, and, with a fully open flap, waits for binding to a new GTP molecule. Our molecular dynamic simulations and rapid flow kinetic studies revealed that the conformational changes in the flap region of RhoA-GDP complex after Tyr34 nitration mimic the conformational shift observed in the flap in a GEF-RhoA complex, whereas GTP binding is unaffected. Therefore, we suggest that nitration of Tyr34 leads to “GEF-like” movement in the flap region while in the GDP-bound state. This will result in faster GDP release and GTP reload, leading to an increase in RhoA activity. Because the introduction of a nitro group on Tyr34 introduces a slight negative charge, we speculate that it may also be a site of physiological regulation of RhoA via phosphorylation. However, whether this occurs, and if so, the identification of the kinases involved will require further study. Tyr34 is subjected to AMPylation (35). Modification of Tyr34 by adenosine monophosphate results in RhoA inhibition, probably because of sterical hindrance of the nucleotide-binding

FIGURE 6. Mechanism of nitration mediated RhoA activation. A, analysis of the x-ray crystal structure of RhoA shows that Tyr34 is located in a flexible regions (flap) of the Switch I domain that is responsible for nucleotide binding. B, a 100-ns molecular dynamics simulation of the flap region in RhoA (blue) and RhoA nitrated at Tyr34 (green) was carried out (left panel); the movement of the flap region (green) was also superimposed onto the x-ray crystal structure RhoA bound to a GEF protein (pink, right panel). His-tagged RhoA construct was expressed in the E. coli BL21 strain. First, the bacterial lysate was purified by His6 tag affinity on a nickel-nitrilotriacetic acid column. Second, the proteins in the elution fraction from the affinity purification were separated by size exclusion chromatography. C, Coomassie staining the purified fraction identified two bands and confirmed as RhoA using Western blot analysis. That both bands are RhoA was further confirmed using MS, suggesting that the second band may contain a post-translational modification. Peroxynitrite decreases GDP binding to purified RhoA. Human recombinant RhoA was exposed to authentic peroxynitrite (100 μM, 30 min) on ice. D and E, the kinetics of binding fluorescently labeled GDP (D) or GTP (E) to RhoA was then performed using stop flow analysis. Three measurements were averaged and fitted with bimolecular reaction equation. Nitration decreases the binding constant of GDP to RhoA from 19.79 ± 1.66 to 4.03 ± 2.87 M−1 s−1 but does not affect GTP binding (RhoA alone = 38.66 ± 8.6 M−1 s−1 versus RhoA + ONOO = 36.19 ± 12.7 M−1 s−1). F, the kinetics of GTP binding to the Y34FRhoA mutant was also measured by stop flow analysis using mant-GTP. No binding of GTP was observed, indicating that this mutant cannot bind GTP and is therefore catalytically inactive. G, the Y34FRhoA mutant protein was overexpressed in HLMVEC. After 48 h there was a significant increase in RhoA protein. H, Y34FRhoA mutant protein overexpression did not alter basal RhoA activity or modulate the increase in RhoA activity in LPS-treated cells. I, the decrease in normalized TER in response to LPS was also not modulated by Y34FRhoA mutant protein overexpression. J, schematic representation of RhoA catalytic cycling. GDP release is a rate-limiting step in the RhoA activation. Increased GDP release is assisted by Tyr34 nitration leading to faster GTP reload and increased RhoA activity. The data are means ± S.E.; n = 3.* p < 0.05 versus pDEST40 (empty vector) no LPS.
site. Further, it is likely that the nitration of Tyr34 would inhibit phosphorylation and/or AMPylation of RhoA. Thus, the competition among these various modifications for one very important site of RhoA likely has both physiological and pathological implications.

To protect RhoA protein from nitration at Tyr34, we utilized the sequence surrounding Tyr34 to construct a novel shielding peptide: NipR1. This sequence was developed based on the observation that multimeric structures occur in RhoA, Rac1, and CDC42 (36, 37). Because we demonstrated a strong affinity between NipR1 and RhoA, we speculate that the flap region may be located at the multimeric interface of RhoA. Binding of the NipR1 peptide prevented nitration of RhoA by shielding protein from peroxynitrite attack. We speculate that the mechanism for this protection is that NipR1 provides an alternative tyrosine residue to react with peroxynitrite rather than simply sterically inhibiting the access of peroxynitrite to the protein. This possibility is supported by our data demonstrating that, despite its ability to bind to RhoA, the NipR1F peptide did not prevent the nitration-mediated activation of RhoA. Interestingly, the NipR1 peptide reduced LPS-mediated RhoA nitration, without affecting other protein nitration events, suggesting that it is targeting RhoA specifically rather than acting as a generic peroxynitrite sink. Further, because NipR1 did not appear to affect basal RhoA activity in either HLMVEC or in mice, we suggest it has potential clinical utility in ALI because even if the peptide gains access to tissues in addition to the lung, it should not alter physiologic RhoA signaling. This is important, because RhoA is also involved in maintaining the endothelial cytoskeleton in a conformation that maintains a tight barrier (38). NipR1 was also efficacious in preventing ALI in mice. In addition to preventing RhoA nitration and activation, NipR1 exerted positive benefits at multiple levels of disease progression. NipR1 preserved endothelial barrier function and attenuated the levels of inflammatory cytokines and chemokines in the BALF. This correlated with the decreased macrophage and neutrophil infiltration into the lungs. It is well established that vascular cells (endothelial and smooth muscle) produce chemokines and cytokines that result in the attraction of neutrophils and macrophages (39, 40). It has been shown that cytokine production in smooth muscle cells correlates with RhoA activation. Thus, we speculate that the NipR1-mediated inhibition of RhoA signaling may also disrupt cytokine production in vascular cells and attenuate the recruitment of neutrophils and macrophages that perpetuate the vicious cycle of immune cell activation. The alveolar structure of the lung was also preserved in NipR1 treated mice, whereas the morphological changes were also reflected in the preservation of lung function. From these data we conclude that our shielding peptide approach is a new and promising therapeutic approach for the management of ALI/ARDS. However, it is worth discussing the barriers for using NipR1 to treat the endothelial injury associated with ALI. Despite decades of ALI research, there are very few therapeutic options beyond low tidal volume mechanical ventilation (41). Further, there are many instances where positive outcomes in animal models of ALI, especially mice, have failed to translate into clinical benefit in humans. For example, recently it was shown that β-adrenergic receptor agonists had positive outcomes in ALI in mice (42–44). However, randomized clinical trials using albuterol showed no improvement in clinical outcomes in patients with ALI (45). Thus, although we present highly positive data regarding the ability of NipR1 to attenuate both the endothelial barrier disruption and the development of ALI in response to LPS, caution should be employed in further extrapolation. Indeed, more studies will be required to determine whether NipR1 can restore the endothelial barrier after LPS exposure and whether it also has a protective effect in other models of ALI such as VILI or in response to live bacterial infections, which more closely resemble the patients presenting with ALI/ARDS.

In conclusion, although previous studies have suggested that the small GTPases can be regulated by cellular redox status (5, 28–30), we are the first to elucidate the molecular mechanisms by which this occurs in response to nitrative stress. We demonstrate that nitration of a single tyrosine residue, Tyr34, is sufficient to increase the activity of RhoA. This involves the induction of a GEF-like conformational change in the flap region of Switch I. Further, for the first time we have utilized a shielding peptide approach to prevent a single targeted protein against post-translational modification by peroxynitrite. Finally, we have demonstrated that a shielding RhoA peptide, NipR1, can prevent nitration of RhoA and preserve the mouse lung at multiple levels against the deleterious effects of LPS. We speculate that peptides based around NipR1 may have clinical utility in the management of ALI/ARDS.

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