**Binding of the Andes Virus Nucleocapsid Protein to RhoGDI Induces
the Release and Activation of the Permeability Factor RhoA**

Short Title: ANDV N Protein Binds RhoGDI to Activate RhoA

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Abstract

Andes virus (ANDV) nonlytically infects pulmonary microvascular endothelial cells (PMECs) causing acute pulmonary edema termed hantavirus pulmonary syndrome (HPS). In HPS patients virtually every PMEC is infected, however the mechanism by which ANDV induces vascular permeability and edema remains to be resolved. The ANDV nucleocapsid (N) protein activates the GTPase, RhoA, in primary human PMECs causing VE-Cadherin internalization from adherens junctions and PMEC permeability. We found that ANDV N protein failed to bind RhoA, but co-precipitates RhoGDI (Rho GDP-dissociation inhibitor), the primary RhoA repressor that normally sequesters RhoA in an inactive state. ANDV N protein selectively binds the RhoGDI C-terminus (69-204) but fails to form ternary complexes with RhoA or inhibit RhoA binding to the RhoGDI N-terminus (1-69). However, we found that ANDV N protein uniquely inhibits RhoA binding to an S34D phosphomimetic RhoGDI mutant. Hypoxia and VEGF increase RhoA induced PMEC permeability by directing Protein Kinase C α (PKC α) phosphorylation of S34 on RhoGDI. Collectively, ANDV N protein alone activates RhoA by sequestering and reducing RhoGDI available to suppress RhoA. In response to hypoxia and VEGF activated PKC α , ANDV N protein additionally directs the release of RhoA from S34-phosphorylated RhoGDI, synergistically activating RhoA and PMEC permeability. These findings reveal a fundamental edemagenic mechanism that permits ANDV to amplify PMEC permeability in hypoxic HPS patients. Our results rationalize therapeutically targeting PKC α and opposing Protein Kinase A (PKA) pathways that control RhoGDI phosphorylation as a means of resolving ANDV induced capillary permeability, edema and HPS.

Importance

HPS causing hantaviruses infect pulmonary endothelial cells causing vascular leakage, pulmonary edema and a 35% fatal acute respiratory distress syndrome (ARDS). Hantaviruses don't lyse or disrupt the endothelium but dysregulate normal EC barrier functions and increase hypoxia directed permeability. Our findings reveal a novel underlying mechanism of EC permeability resulting from ANDV N protein binding to RhoGDI, a regulatory protein that normally maintains edemagenic RhoA in an inactive state and inhibits EC permeability. ANDV N sequesters RhoGDI and enhances the release of RhoA from S34 phosphorylated RhoGDI. These findings indicate that ANDV N induces the release of RhoA from PKC phosphorylated RhoGDI, synergistically enhancing hypoxia directed RhoA activation and PMEC permeability. Our data suggests inhibiting PKC and activating PKA phosphorylation of RhoGDI as mechanisms of inhibiting ANDV directed EC permeability and therapeutically restricting edema in HPS patients. These findings may be broadly applicable to other causes of ARDS.

Introduction

Hantaviruses nonlytically infect nearly every pulmonary microvascular endothelial cell (PMEC) in the lung⁽¹⁾, causing acute pulmonary edema that leads to respiratory insufficiency termed hantavirus pulmonary syndrome (HPS)⁽¹⁻⁷⁾. HPS is fatal in 35-40% of cases and characterized by thrombocytopenia, hypoxia, increased vascular permeability and pulmonary edema resulting in the rapid onset of acute respiratory distress^(1, 5, 6, 8-11). HPS causing hantaviruses include Andes virus (ANDV), Sin Nombre virus (SNV) and others that are uniquely present in the Americas^(10, 12-17). ANDV is the only hantavirus reported to spread person to person^(14, 15) and to cause lethal HPS-like disease in immunocompetent Syrian hamsters⁽¹⁸⁻²²⁾.

The unique endothelial cell tropism of hantaviruses and the failure of hantavirus infections to disrupt the endothelium, reflect hantavirus dysregulation of PMEC barrier functions that are critical to capillary leakage and HPS disease^(1, 3-6). Pathogenic hantaviruses engage inactive, bent, $\alpha_v\beta_3$ integrin conformers in order to infect PMECs⁽²³⁻²⁶⁾, and hantaviruses remain cell associated^(27, 28), recruiting inactivated platelets to EC surfaces^(27, 29, 30). In addition, PMECs infected by pathogenic hantaviruses are hyperresponsive to vascular endothelial growth factor (VEGF)⁽²⁹⁻³²⁾, a vascular permeability factor induced by hypoxia, that causes edema and is elevated in hypoxic HPS patient pulmonary edema fluids^(31, 33). However, mechanisms by which hantaviruses dysregulate PMEC barrier functions remain to be resolved.

The primary fluid barrier of capillaries is maintained by adherens junctions (AJs) composed of interendothelial cell complexes of VE-cadherin⁽³⁴⁻³⁷⁾. Intracellularly, VE-cadherins engage the actin cytoskeleton and are dynamically regulated by signaling pathways that control cell morphology, angiogenesis, leukocyte extravasation and paracellular permeability^(34-36, 38). The cellular GTPases Rac1 and RhoA opposingly regulate the density of VE-cadherin in AJs, immune cell diapedesis and the fluid barrier integrity of the endothelium^(34, 39-45). Rac1 activation stabilizes AJs while RhoA activation

dissociates and internalizes VE-cadherin from AJs increasing capillary permeability^(34, 35, 37, 38, 41, 42, 44, 46-49).

RhoA is maintained in an inactive GDP-bound form by constitutively binding to Rho guanine dissociation inhibitor (RhoGDI)⁽⁵⁰⁻⁵⁸⁾. RhoGDI sequesters RhoA in the cytoplasm preventing RhoA translocation to the plasma membrane and the formation of activated RhoA-GTP complexes^(50, 54, 59). There are 3 mammalian RhoGDI isoforms and the most abundant form, RhoGDI α , binds to RhoA, Rac1 or Cdc42 GTPases that when activated, positively or negatively impact EC barrier functions^(50, 54, 56). RhoGDI α has 2 domains, an N- terminal domain (residues 5-55) that mediates GTPase binding, and a C-terminal immunoglobulin-like domain (AAs 74-204) that includes a Rho family prenyl-group binding pocket^(50, 54, 60). RhoGDI is regulated by site-specific phosphorylation (S34/S96/S174) that determines its ability to sequester specific GTPases in an inactive state^(48, 53-58). Protein kinase C (PKC) phosphorylation of RhoGDI α □□ serine 34 is reported to cause a specific decrease in RhoGDI affinity for RhoA, but not Rac1 or Cdc42⁽⁵¹⁻⁵⁴⁾. In contrast, Protein kinase A phosphorylation of S174 selectively releases Rac1 from RhoGDI, negatively regulates RhoA activation and stabilizes EC barriers⁽⁶¹⁻⁶⁵⁾. As a result, targeted RhoGDI phosphorylation by upstream signaling pathways selectively enhances RhoA release from RhoGDI, RhoA activation and EC permeability⁽⁵³⁻⁵⁵⁾. The critical role of RhoGDI in RhoA directed vascular leakage and ARDS is also demonstrated by conditional RhoGDI knockouts that alone are sufficient to disrupt murine pulmonary vascular barriers, increase basal PMEC permeability and cause pulmonary edema^(44, 55, 56, 66, 67).

ANDV infection results in RhoA activation and PMEC hyperpermeability in response to hypoxia or VEGF addition^(68, 69). Knockdown of RhoA, expression of dominant negative-RhoA, or inhibiting RhoA kinase was found to reduce ANDV directed PMEC permeability⁽⁷⁰⁾. In addition, we found that expressing the ANDV N protein, converts RhoA into an active GTP-bound form that increases PMEC permeability⁽⁷⁰⁾. However, the mechanism by which the ANDV N protein activates RhoA and augments basal PMEC vascular permeability responses remain to be defined.

118 In this report we evaluated ANDV N protein interactions with RhoA and RhoGDI which control
119 PMEC permeability. Our results indicate that ANDV N protein fails to directly interact with RhoA, and
120 instead binds to the RhoA inhibitor RhoGDI. In contrast to nonpathogenic TULV N protein, we found
121 that ANDV N protein dose dependently co-precipitates RhoGDI by binding to the C-terminal domain of
122 RhoGDI. However, ANDV N failed to bind the N-terminus of RhoGDI or disrupt N-terminal binding of
123 RhoGDI to RhoA. Thus at a basal level, RhoA and ANDV N bind non-competitively to discrete
124 RhoGDI protein complexes. ANDV N protein is phosphorylated on S386, however we observed no
125 change in RhoA binding to RhoGDI in the presence of ANDV N-S386A or N-S386D phosphorylation
126 mutants⁽⁷¹⁾. Hypoxia and VEGF activate RhoA by directing PKC phosphorylation of S34 on RhoGDI. In
127 contrast to findings that ANDV N fails to direct basal RhoGDI release of RhoA, we observed that co-
128 expressing ANDV N protein prevented RhoA binding to a phosphomimetic RhoGDI-S34D mutant.
129 These findings suggest that ANDV N protein directs the release of RhoA from phosphorylated RhoGDI,
130 amplifying RhoA activation directed by hypoxia and VEGF. This data suggests inhibiting PKC
131 phosphorylation of RhoGDI-S34 and activating PKA directed RhoGDI-p174 as a potential therapeutic
132 mechanism of blocking ANDV directed EC permeability, edema and HPS.

Results

Hantaviruses predominantly infect the EC lining of capillaries and in HPS patients nearly every pulmonary MEC is hantavirus infected^(1, 3). ANDV infection of PMECs, exposure of infected PMECs to VEGF or hypoxia, and expression of ANDV N protein in PMECs increases the permeability of interendothelial cell adherens junctions by activating RhoA^(30, 32, 68-70). The mechanism by which ANDV N protein activates RhoA and induces capillary permeability remains to be defined and is likely to suggest therapeutic approaches for inhibiting ANDV induced edema and HPS.

ANDV N Protein Binds RhoGDI, not RhoA

We previously reported that ANDV infection or N protein expression in PMECs activates RhoA and directs PMEC permeability⁽⁷⁰⁾. To determine if ANDV N protein binds RhoA or its constitutive inhibitor protein, RhoGDI, we co-transfected HEK293T cells with plasmids expressing Flag-tagged ANDV N protein, HA-tagged RhoGDI and Myc-tagged RhoA. ANDV N protein was immunoprecipitated with anti-Flag-tag antibody and assayed for co-precipitated RhoGDI and RhoA by Western blot. We found that ANDV N protein selectively co-precipitated RhoGDI but failed to co-precipitate RhoA in the presence or absence of RhoGDI (Figure 1A). We found that dose dependent increases in ANDV N resulted in a concomitant increase in co-precipitated RhoGDI (Figure 1 B) and reciprocally that increased in RhoGDI expression enhanced co-precipitation of ANDV N protein (Figure 1 C). These findings suggest that ANDV N protein selectively binds and sequesters RhoGDI, preventing RhoGDI binding and suppression of RhoA.

ANDV, but not TULV, N Protein Binds RhoGDI and Activates RhoA

ANDV is an HPS causing hantavirus that induces PMEC permeability, while TULV is a nonpathogenic hantavirus that infects PMECs but lacks the ability to activate RhoA or permeabilize PMEC monolayers^(29, 30, 69, 70, 72). Here we comparatively analyzed the ability of ANDV and TULV N proteins to bind RhoGDI and activate RhoA. While ANDV N protein robustly co-precipitated RhoGDI from HEK cell lysates, we found that TULV N protein failed to co-precipitate RhoGDI (Figure 2A). In

order to examine N protein binding to endogenous RhoGDI present in PMECs, we lentivirus transduced and puromycin selected PMECs expressing ANDV or TULV N proteins. Immunoprecipitating endogenous RhoGDI from PMECs resulted in the co-precipitation of ANDV N protein, but not TULV N protein (Figure 2B). In addition, we found that RhoA was activated (RhoA-GTP) in PMECs expressing the ANDV N protein but not in PMECs expressing TULV N (Figure 2C). These findings demonstrate that N protein from HPS causing ANDV, but not nonpathogenic TULV, binds to endogenous RhoGDI and activates RhoA in PMECs. These findings link ANDV N protein binding to RhoGDI to the selective activation of RhoA during ANDV infection⁽⁷⁰⁾.

ANDV N Protein Dose Dependently Binds RhoGDI Independent of RhoA Expression.

Our data suggests that RhoGDI binds RhoA independent of binding to ANDV N protein. To determine whether ANDV N and RhoA proteins bind competitively to RhoGDI, we assayed RhoGDI binding in the presence of increasing amounts of RhoA or N proteins. We found that increased expression of RhoA failed to alter N protein co-precipitation of RhoGDI (Figure 3A) or RhoGDI co-precipitation of ANDV N protein (Figure 3B). In addition, expressing an increasing amount of ANDV N protein failed to impact RhoGDI co-precipitation of RhoA (Figure 3C). To confirm this we reciprocally co-precipitated RhoA or RhoGDI and evaluated components of RhoGDI complexes in the presence or absence of N protein. The presence of co-expressed ANDV N protein failed to alter RhoA co-precipitation of RhoGDI (Figure 4A), or RhoGDI co-precipitation of RhoA (Figure 4B). Consistent with this RhoA co-precipitated RhoGDI, but failed to co-precipitate N protein, and reciprocally N protein co-precipitated RhoGDI but not coexpressed RhoA (Figure 4C). These findings confirm that RhoA and ANDV N protein fail to interact, and demonstrate that RhoGDI forms discrete complexes with ANDV N protein and RhoA, without competition for RhoGDI binding sites or the formation of ternary complexes.

ANDV N Binds to the C-terminus of RhoGDI

The switch II domain present in the N-terminus of RhoGDI (AAs 1-69) reportedly binds RhoA and regulates RhoA activation^(54, 73). We expressed N-terminal (1-69) and C-terminal (69-204) domains

of RhoGDI and defined requirements for ANDV N protein binding. We found that ANDV N protein co-precipitated the RhoGDI C-terminus (Figure 5A) but failed to bind the N-terminal domain of RhoGDI (Figure 5B). These findings are consistent with the absence of competitive binding by RhoA and N protein to RhoGDI and indicate that RhoA and ANDV N bind discrete N-terminal and C-terminal domains of RhoGDI, but lack the ability to form RhoA-RhoGDI-N protein ternary complexes. Collectively these findings indicate that N protein sequesters RhoGDI in complexes that lack the ability to bind RhoA, and are consistent with N protein reducing the amount of RhoGDI available to suppress RhoA activation.

ANDV N Protein Phosphorylation Does not Impact RhoGDI binding or Phosphorylation

cAMP activated protein kinase A (PKA) directs RhoGDI-S174 phosphorylation which stabilizes RhoGDI binding to Rho family kinases and could impact N protein binding to RhoGDI^(51, 52, 54, 61, 64, 65, 74-78). In addition, ANDV N protein is phosphorylated on S386⁽⁷¹⁾, and the phosphorylation of ANDV N protein could impact its binding to RhoGDI or PKA directed phosphorylation of RhoGDI. We expressed S386A, S386D and S386H mutants of ANDV N protein and determined if mutant N proteins bind to RhoGDI or alter RhoGDI-S174 phosphorylation. In cells expressing WT or S386 mutant N proteins, we found no change in the ability of RhoA to co-precipitate RhoGDI (Figure 6A). Similarly, we found that ANDV N S386-A, -D or -H mutants failed to alter RhoGDI phosphorylation of S174, using a phosphospecific RhoGDI-^PS174 antibody, (Figure 6B).

ANDV N Protein Inhibits RhoA Binding to the Phosphomimetic RhoGDI-S34D Protein

Hypoxia and VEGF activate PKC α which phosphorylates RhoGDI on serine residues S34 and S96 and regulates RhoA activation⁽⁵³⁾. PKC phosphorylation of RhoGDI-S34 reportedly reduces RhoGDI affinity for RhoA, but not Rac1 or Cdc142 Rho family GTPases^(53, 56), and increases EC permeability^(53, 55, 79-81). We expressed RhoGDI S34A/S34D and S96A/S96D mutants and found no effect on RhoGDI co-precipitation of RhoA by S34A, S96A or S96D mutants, while the RhoGDI-S34D mutant partially reduced RhoA co-precipitation (Figure 7A). Consistent with N binding to the RhoGDI

208 C-terminus, we found no difference in the ability of ANDV N protein to co-precipitate RhoGDI-S34A
 209 or -S34D mutants (Figure 7B). However, in the presence of co-expressed N protein, we found a nearly
 210 complete inhibition of RhoA co-precipitation by RhoGDI-S34D, which is not observed from co-
 211 precipitating WT or S34A RhoGDI (Figure 7C). These results indicate that ANDV N protein uniquely
 212 inhibits RhoA binding to RhoGDI-S34D, a RhoGDI-^PS34 phosphomimetic^(53, 80-82). This suggests that
 213 the basal ability of ANDV N protein to bind RhoGDI and activate RhoA is enhanced in the presence of
 214 PKC phosphorylated RhoGDI (S34D), completely blocking RhoGDI binding to RhoA. This reveals a
 215 novel mechanism for N protein to synergistically enhance RhoA activation and EC permeability induced
 216 by hypoxia and VEGF, that direct PKC phosphorylation of RhoGDI-^PS34. These results suggest the
 217 potential of inhibiting PKC phosphorylation of RhoGDI as a means of therapeutically reducing N
 218 protein directed RhoA activation and PMEC permeability during ANDV infection.

Discussion

Endothelial cells are the primary cellular targets of hantavirus infection, and in HPS patients nearly every pulmonary microvascular endothelial cell (PMEC) is nonlytically infected^(1, 3, 5, 11). ECs regulate vascular barrier functions through unique receptors, adherens and tight junction proteins and signaling responses that are in place to maintain hemostasis^(1, 35, 83-91). The novel tropism of HPS-causing hantaviruses for PMECs, and the role of PMECs in regulating pulmonary edema, has focused studies on hantavirus dysregulation of PMEC functions that increase vascular permeability^(7, 29, 31, 70, 89, 92-94). Several facets that control capillary permeability are impacted by pathogenic hantavirus infection of PMECs and contribute to HPS edema. Pathogenic hantaviruses bind and inhibit the function of β_3 integrins on PMECs and platelets, the only integrins directly linked to vascular leakage^(23, 24, 27, 29). Following PMEC infection, hantaviruses remain bound to cell surface β_3 integrins where they recruit platelets to infected PMECs without platelet activation^(23-26, 90, 91, 95). HPS is an acute respiratory distress syndrome (ARDS) that causes hypoxia and induces the secretion of VEGF, a vascular permeability factor that is elevated in HPS patient edema fluids^(7, 29, 30, 33, 69). Consistent with this PMECs infected by pathogenic hantaviruses are hyper-permeabilized by hypoxic conditions and VEGF^(27, 29, 30, 32, 33, 92, 96).

Capillary permeability directed by VEGF, thrombin, bradykinin and $\text{TNF}\alpha$ is mediated by the activation of RhoA^(40, 42, 97-102). RhoA activation disrupts fluid barrier functions of PMEC by directing the disassembly of VE-cadherin within interendothelial adherens junctions and increases capillary permeability^(36, 38, 47, 103-106). RhoGDI is a constitutive inhibitor of RhoA activation that normally prevents PMEC permeability and pulmonary edema^(48, 50, 53-57, 67, 81, 107). RhoGDI binds RhoA in the cytoplasm of PMECs and prevents RhoA activation and RhoA prenyl group attachment to the plasma membrane^(50, 54, 59).

We previously found that infection of primary human PMECs by HPS-causing ANDV activates RhoA and induces PMEC permeability^(29, 30, 69, 70). In contrast PMECs infected with TULV, a nonpathogenic hantavirus, fail to induce PMEC permeability or RhoA activation⁽⁷⁰⁾. Further analysis

demonstrated that the singular expression of the N protein from ANDV, but not TULV, activated RhoA and that inhibitors of RhoA or RhoA kinase (ROCK) block ANDV induced permeability of PMECs⁽⁷⁰⁾. Findings presented here demonstrate that the ANDV N protein uniquely binds to RhoGDI and sequesters RhoGDI in complexes incapable of binding RhoA. In addition to RhoA binding, ANDV N protein specifically enhanced RhoA release from S34 phosphorylated RhoGDI, suggesting that signaling pathway specific RhoA activation is synergistically amplified by ANDV N binding to RhoGDI.

In addition to binding to RhoA, RhoGDI also binds to RhoGTPases Rac1 and Cdc42, with discrete release of GTPases by pathway and site specific RhoGDI phosphorylation^(50-52, 54, 56-58, 65, 81, 108-110). However, unlike RhoA activation, Rac1 activation is associated with enhanced EC barrier functions that counter and oppose the effects of RhoA directed EC permeability^(34, 41, 45, 46, 51, 55, 61, 65, 106). In addition, there are many cell signaling pathways that act on RhoGDI to positively or negatively control RhoA or Rac1 activation^(50, 52-56, 58, 111, 112). Thus roles for RhoGDI in regulating RhoGTPase responses are complicated by signaling pathway phosphorylation of RhoGDI that results in the selective release of RhoA or Rac1, and controls the balance of barrier integrity and permeability^(50, 52-56). RhoGDI is reportedly phosphorylated on S34, S96, S101 and S174 with discrete interactions and GTPase activation responses directed by each^(50, 52, 54, 56, 58, 73, 110). P-21 Activated Kinase (PAK1) reportedly phosphorylates S101 and S174 residues on RhoGDI selectively reducing affinity for Rac1, but not RhoA, and resulting in Rac1 activation and enhanced EC barrier function^(51, 52, 54, 58, 61, 63, 76, 110). In contrast, Protein Kinase C α (PKC α) phosphorylation of S34 on RhoGDI selectively reduces binding to RhoA, but not Rac1 or Cdc42, and promotes RhoA activation and EC permeability^(53-56, 79, 81, 82, 113). PKC α phosphorylation of S96 is reported to release both RhoA and Rac1 from RhoGDI^(50, 54, 56).

PKC α phosphorylates S34 in the N-terminal regulatory arm of RhoGDI that interacts with the switch II domain of RhoA^(53, 54, 81). Given roles for PKC α phosphorylated RhoGDI in selectively regulating RhoA, we determined whether ANDV N protein binding to RhoGDI was altered by mutating S34A, S96A, S34D or S96D phosphorylation sites or whether N protein inhibited RhoGDI binding to

269 RhoA. Consistent with prior studies⁽⁵³⁾, we observed a partial decline in RhoA binding to the
 270 phosphomimetic RhoGDI S34D mutant in the absence of ANDV N protein (Fig 7A) and we found no
 271 difference in ANDV N protein binding to RhoGDI phosphorylation site mutants (Fig. 7B). However, in
 272 the presence of ANDV N protein we found that binding of RhoA to the RhoGDI S34D mutant was
 273 nearly completely abolished. While ANDV N protein binding to RhoGDI does not compete with a
 274 RhoA binding site, these findings demonstrate that N protein binding to phosphomimetic RhoGDI S34D
 275 dramatically reduces the ability of the RhoGDI N-terminal domain to bind and sequester RhoA in an
 276 inactive state. This finding explains how ANDV N protein binding to RhoGDI-^PS34 enhances RhoA
 277 release from its constitutive inhibitor and activates RhoA. These results define a novel mechanism of
 278 ANDV induced PMEC permeability, and suggest inhibiting PKC α phosphorylation of RhoGDI as a
 279 means of restricting ANDV activation of RhoA and PMEC permeability during HPS.

280 Hantavirus N proteins are abundantly expressed during infection and have the potential to bind
 281 and sequester PMEC proteins. We found that ANDV N protein binds to the N-terminal (1-69) domain of
 282 RhoGDI, but have yet to define determinants of ANDV N protein binding to RhoGDI. Overall N
 283 proteins are antigenically conserved across diverse hantaviruses circulating worldwide. However, the N
 284 protein from HPS-causing ANDV (South American) is only 75% identical with the nonpathogenic
 285 TULV (European). ANDV and TULV differ by a large hypervariable domain (residues 249-304; only
 286 31% amino acid conservation) and a novel S386 phosphorylation site (TULV S386E) that in ANDV N
 287 protein regulates IFN responses⁽⁷¹⁾. However, we found no difference in the binding of RhoGDI to WT
 288 or ANDV N protein S386A/D/H mutants (Fig. 6). It remains to be discovered what confers ANDV N
 289 protein binding to RhoGDI, whether N proteins from other HPS or HFRS causing hantaviruses
 290 commonly bind RhoGDI, or whether N protein signatures that direct RhoGDI binding are uniquely
 291 associated with HPS.

292 The role of RhoGDI in ANDV induced edema is tied to in vivo endothelial cell proliferative and
 293 permeability responses. RhoGDI deficiency induces constitutive activation of RhoGTPases and is a

critical mechanism of breast cancer tumor development^(54, 67, 112, 114, 115). Loss of RhoGDI in mice results in progressive renal defects, and PKC regulated RhoGDI responses control RhoA directed permeability of brain MECs and the blood brain barrier^(79, 113, 116). In mice, knocking out RhoGDI activates RhoA, opens interendothelial junctions in lung microvessels and has a net effect of increasing capillary permeability that by itself causes pulmonary edema in vivo^(44, 53, 55, 56, 81, 117). These findings demonstrate the fundamental importance of PKC and RhoGDI in controlling endothelial cell permeability, and ARDS, that may be therapeutically targeted.

Causes of ANDV directed PMEC dysfunction resulting in vascular permeability have been difficult to define due to the complexity of endothelial cell and platelet responses in hemostasis, redundant roles of PMEC permeability inducers and regulators, and the difficulty of studying EC responses in vivo that are magnified in ABSL4 settings. Prior studies have shown that ECs infected by pathogenic, but not nonpathogenic hantaviruses, are hyperpermeabilized by VEGF addition or by hypoxic conditions observed at late stages of HPS^(29, 30, 32, 69, 105, 118). HPS-causing hantaviruses appear to enhance RhoA activation through several mechanisms including VEGF and inhibiting $\alpha_v\beta_3$ integrin and FAK signaling responses that normally activate Rac1^(25, 27, 28, 30, 32, 33, 44, 49, 69, 70, 79, 119-121). Given the fundamental role of RhoGDI in RhoA activation, PMEC permeability and pulmonary edema, our findings suggest RhoGDI regulated responses are central to RhoA activation and permeability responses during ANDV infection.

Findings presented here demonstrate a role for ANDV N protein-RhoGDI complexes directing RhoA activation that is enhanced by VEGF induced PKC phosphorylation^(79, 113, 122, 123). In the endothelium, VEGF directed PKC phosphorylation of RhoGDI is opposed and countered by protein kinase A (PKA) directed phosphorylation that maintains vascular hemostasis^(50, 52-56, 58). PKA reportedly phosphorylates RhoA on S188 and RhoGDI on S174 and both events are linked to increased RhoGDI binding to RhoA, the selective release of Rac1 and inhibited EC angiogenesis and permeability responses^(50, 52, 54, 57, 58). PKA inhibitors induce intercellular gaps between ECs, and PKA also activates

eNOS directed vasodilation, by phosphorylating S1177 and directing T495 dephosphorylation^(54, 124). Opposing PKA regulation, VEGF directs PKC phosphorylation of S34 on RhoGDI and inhibits eNOS activity by phosphorylating T495 and dephosphorylating S1177^(54, 124). The ability of ANDV N to enhance RhoA release from S34D-RhoGDI provides a rationale for the increased permeability of ANDV infected PMECs in response to hypoxia/VEGF and is consistent with PKC phosphorylation of RhoGDI-^PS34. As a result, N protein binding to RhoGDI provides a novel mechanism of amplifying VEGF directed PMEC permeability during HPS.

Our findings are consistent with hantavirus N protein interactions with RhoGDI directing one part of a collective ARDS program of hypoxia directed VEGF responses that synergize with RhoGDI regulation, RhoA activation and the inhibition of $\beta 3$ integrin and platelet functions during HPS. Currently there are no therapeutic approaches for treating hantavirus induced HPS or HFRS diseases, or reducing the lethal outcome of symptomatic HPS patients⁽¹²⁵⁾. Our findings provide a mechanism for HPS-causing ANDV N protein to engage RhoGDI and fundamentally alter the regulation of RhoA, a central inducer of PMEC permeability and angiogenic repair^(50, 53, 54, 110). We previously reported that in vitro, ANDV directed PMEC permeability is dramatically reduced by pharmacologic RhoA/ROCK inhibitors^(29, 30, 32, 70). Our new findings add to this by suggesting the potential for activating PKA and PAK1, and inhibiting PKC as a means of controlling RhoGDI-RhoA complexes and restoring RhoGDI suppression of RhoA. We found that VEGFR inhibitors and FTY720, a PAK1 activator, reduce hantavirus directed EC permeability^(29, 30), suggesting that activating PAK1 may synergistically enhance the effect of RhoA inhibitors in blocking PMEC permeability. Activating PKA with cAMP-elevating prostaglandins, rolipram, forskolin, atrial natriuretic peptide or cAMP analogs^(62, 63, 65, 75-77, 126-129) have yet to be tested as inhibitors of ANDV permeability but have the potential to counter effects of VEGF directed PKC activation and instead foster Rac1 barrier functions, and inhibit RhoA activation. These findings rationalize the further analysis of pathway specific regulators of RhoGDI as a means of resolving ANDV directed HPS in an ABSL4 Syrian hamster disease model^(18, 22). Our findings describe

344 a novel mechanism of ANDV induced PMEC permeability that gives rise to a 35% fatal ARDS in HPS
345 patients. Although our studies are focused on resolving parameters of ANDV induced ARDS, the central
346 role of RhoGDI and RhoA in vascular permeability, PMEC repair and hemostasis suggests the potential
347 for pathway specific RhoGDI effectors to be broadly applicable to other viral causes of ARDS.

Materials and Methods

Cells and Virus VeroE6 cells and HEK239T cells were grown in DMEM, 10% FCS, antibiotics as previously described. Primary human pulmonary microvascular ECs (PMECs) were purchased from Lonza Inc. (cc-2527), grown in endothelial growth medium-2MV (EGM-2MV; Lonza) and supplemented as previously described^(29, 30, 70). Primary human PMECs were transduced with lentiviruses expressing ANDV or TULV N proteins, puromycin selected and assayed for N protein expression by Western blot (WB).

Plasmids and Constructs Plasmids expressing myc-tagged RhoA constructs were previously described. HA-tagged RhoGDI (ARHGDI; pJP1520) was purchased from Arizona State University/DNASU Plasmid Repository. HA-tagged RhoGDI truncations RhoGDI-1-69 and HA-RhoGDI-69-204, were respectively generated by inserting a termination codon after residue 69 by site-directed mutagenesis of pLenti-puro HA-RhoGDI plasmid, or by PCR cloning into BamHI-XbaI sites of pLenti-puro plasmids. Flag-tagged ANDV and TULV N protein ORFs were inserted BamHI-XbaI into pLenti-CMV-GFP-Puro vector as previously described^(70, 71). Lentiviruses were generated by co-transfecting HEK293T cells with pLenti-purovectors and 3rd generation lentiviral packaging plasmids⁽¹³⁰⁻¹³²⁾ p-RSV-Rev, pMD.2G, pMDLg/pRRE (Addgene 658-5, 12259, 12251, 12253) to generate lentiviruses expressing ANDV or TULV N proteins. Passage 3 PMECs were transduced with recombinant ANDV-N or TULV-N protein expressing lentiviruses at an MOI of 5, initially puromycin selected (0.3-0.5 µg/ml) and passed in the absence of puromycin prior to studies at passage 6-7. N protein expression was detected in >95% of transduced MECs by immunoperoxidase staining and Western blot of PMECs using anti-N polyclonal antibodies as previously described^(29, 30, 70). HEK293 cells were calcium phosphate or PEI transfected with expression plasmids as previously described^(30, 70, 133).

Antibodies Mouse monoclonal antibodies to RhoA, RhoGDI, HA-Tag, Myc-Tag were purchased from Santa Cruz (sc-418, sc-13120, sc-7392, sc-40). Rabbit antibodies to RhoA, RhoGDI, HA-Tag,

Myc-Tag, Flag-Tag were purchased from Cell Signaling (2368, 2272, 2117, 2564) and mouse antibodies to actin, and Flag-Tag were from Sigma (F2426), and Phospho-RhoGDI (Ser174) was from Invitrogen (PA5 38827). Anti-N protein polyclonal rabbit sera made to NY-1V N protein was previously described. Analysis of activated RhoA-GST was performed using GST-Rhotekin-RBD from Cytoskeleton Inc⁽⁷⁰⁾.

Co-immunoprecipitation and Western Blot

Briefly, cells were lysed in buffer containing

1% NP-40 (150 mM NaCl, 40 mM Tris-Cl, 10% glycerol, 2 mM EDTA, 10 nM sodium fluoride, 2.5 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM β -glycerophosphate) with protease inhibitor cocktail (Sigma)⁽¹³⁴⁾. Total protein levels were determined and 20 μ g of protein was resolved by SDS-polyacrylamide (10 %) gel electrophoresis. Co-immunoprecipitations were performed in 1% NP40 lysis buffer as previously described⁽¹³⁴⁾ using indicated antibodies incubated overnight followed by Protein A/G agarose precipitation (sc-2003), three washes in lysis buffer and resuspension in SDS sample buffer prior to SDS gel electrophoresis and Western blot (WB) analysis as previously described^(30, 70). Proteins were transferred to nitrocellulose, blocked in 2% bovine serum albumin, and incubated with indicated antibodies and detected using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL reagent (Amersham) as previously described^(30, 70).

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400

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Figure Legends

Figure 1 ANDV N protein Co-precipitates RhoGDI, but Fails to Co-IP RhoA

A. HEK293T cells were co-transfected with plasmids expressing Flag-tagged ANDV N protein, HA-tagged RhoGDI and Myc-tagged RhoA. Cell lysates were immunoprecipitated with anti-Flag antibody and assayed by WB of co-precipitated RhoGDI or RhoA and assayed for input protein expression⁽⁷⁰⁾. **B.** HEK293T cells were co-transfected with increasing amounts of plasmid expressing the Flag-tagged N protein and constant amounts of HA-tagged RhoGDI. Cell lysates were immunoprecipitated with anti-Flag antibody and evaluated for immunoprecipitated RhoGDI. **C.** HEK293T cells were co-transfected with increasing amounts of plasmid expressing HA-tagged RhoGDI and a constant amount of Flag-tagged N protein. Cell lysates were immunoprecipitated with anti-HA antibody and evaluated for co-precipitated N protein by WB. Expressed input protein and cellular actin protein controls were assayed by WB.

Figure 2 ANDV, but not TULV, N protein Binds RhoGDI and Activates RhoA

A. HEK 293T cells were transfected to express HA-tagged RhoGDI, and Flag-tagged ANDV or TULV N proteins as in Figure 1. Cell lysates were immunoprecipitated with Flag-tag antibody and assayed for co-precipitated HA-tagged RhoGDI protein by WB. **B.** Primary human PMECs were transduced to express Flag-tagged ANDV or TULV N protein. Cell lysates were immunoprecipitated with antibody to RhoGDI and assayed for co-precipitated N proteins by WB. **C.** Control primary human PMECs and PMECs transduced to express ANDV or TULV Flag-N proteins were assayed for RhoA activation (RhoA-GTP) using the GST-Rhotekin-RBD assay and by WB for RhoA and Flag-tagged N protein expression⁽⁷⁰⁾. Expressed input protein and cellular actin protein controls were assayed by WB.

Figure 3 Increasing RhoA or ANDV N Protein Expression Does Not Alter Binding to RhoGDI

A,B. HEK293T cells were co-transfected with increasing amounts of Myc-tagged RhoA plasmid and constant amounts of Flag-tagged ANDV N and HA-tagged RhoGDI proteins. **A)** Cell lysates were

immunoprecipitated with anti-Flag antibody and evaluated for co-immunoprecipitated RhoGDI by WB; or **B**) immunoprecipitated with anti-HA antibodies and evaluated for co-precipitated ANDV N protein by WB. **C.** HEK293T cells were co-transfected with increasing amounts of a plasmid expressing the Flag-tagged ANDV N protein and constant amounts of myc-RhoA and HA-tagged RhoGDI plasmids. Cell lysates were immunoprecipitated with anti-Flag antibody and co-precipitated RhoGDI was evaluated by WB. Expressed input protein and cellular actin protein controls were assayed by WB.

Figure 4. RhoGDI Binding of RhoA is Independent of RhoGDI Binding to N Protein. HEK293T cells were co-transfected with plasmids expressing Flag-tagged ANDV N protein, HA-tagged RhoGDI and Myc-tagged RhoA. **(A)** Cell lysates were immunoprecipitated with anti-Myc antibody and assayed by WB for co-precipitated RhoGDI. **(B)** Cell lysates were immunoprecipitated with anti HA-tag antibodies and assayed for co-precipitation of Myc-RhoA by WB. **(C)** Cell lysates were immunoprecipitated with anti-Myc antibodies and assayed for co-precipitated Flag-N protein or HA-RhoGDI by WB (upper panel). Cell lysates were immunoprecipitated with anti-Flag antibodies and assayed for co-precipitated Myc-RhoA and HA-RhoGDI by WB. Expressed input protein and cellular actin protein controls were assayed by WB.

Figure 5 ANDV N protein Binds RhoGDI C-terminal Domain (69-204). HEK293T cells were co-transfected with plasmids expressing Flag-tagged ANDV N protein, full-length HA-tagged RhoGDI and an HA-tagged C-terminal domain of RhoGDI containing residues 69 to 204 **(A)** or an HA-tagged N-terminal RhoGDI domain containing residues 1-69 **(B)**. Cell lysates were immunoprecipitated with anti-Flag antibody and assayed for co-precipitated full-length HA-RhoGDI or truncated HA-RhoGDI-69-204 or HA-RhoGDI-1-69 by WB. Expressed input protein and cellular actin protein controls were assayed by WB.

Figure 6 ANDV N S386 mutants Fail to Inhibit RhoA Binding or RhoGDI Phosphorylation. HEK293T cells were co-transfected with plasmids expressing HA-tagged RhoGDI, Myc-tagged RhoA,

455 Flag-tagged ANDV N or ANDV N protein mutants (S386A, S386D or S386H)(71). **A)** Cell lysates were
 456 immunoprecipitated with anti-Myc antibody and assayed for co-precipitated RhoGDI by WB. **B)**
 457 HEK293T cells were co-transfected with plasmids expressing HA-tagged RhoGDI, and Flag-tagged
 458 ANDV N or ANDV N protein mutants (S386A, S386D or S386H). Cell lysates were assayed by WB
 459 for changes in the phosphorylation of RhoGDI using a phospho-specific antibody to S174 as well as for
 460 input levels of total RhoGDI protein and WT or S386 mutants of ANDV N protein.

461 **Figure 7 Phosphomimetic S34D RhoGDI Mutant decreased affinity for RhoA.**

462 **A)** HEK293T cells were co-transfected with plasmids expressing constant amounts of Myc-tagged
 463 RhoA and HA-tagged RhoGDI WT or mutant (S34A, S34D, S96A, S96D). Cell lysates were
 464 immunoprecipitated with anti-HA antibody and assayed for co-precipitated RhoA and RhoGDI proteins
 465 by WB. **B)** HEK293T cells were co-transfected with plasmids expressing ANDV N protein and HA-
 466 tagged RhoGDI WT or RhoGDI-S34A, or -S34D mutants. Cell lysates were immunoprecipitated with
 467 anti-Flag antibody and assayed for co-precipitated RhoGDI proteins. **C)** HEK293T cells were co-
 468 transfected with plasmids expressing ANDV N protein, Myc-RhoA and HA-tagged RhoGDI WT or
 469 RhoGDI-S34A, or -S34D mutants. Cell lysates were immunoprecipitated with anti-HA antibody and
 470 assayed for co-precipitated RhoA by WB. Expressed input protein and cellular actin protein controls
 471 were assayed by WB.

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Figure 1

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