Hyaluronic Acid Binding Protein 2 Is a Novel Regulator of Vascular Integrity

N. Mambetsariev; T. Mirzapoiazova; B. Mambetsariev; S. Sammani; F.E. Lennon; J.G.N. Garcia; Patrick A. Singleton

Background and Purpose—We evaluated the role of the extracellular serine protease, hyaluronic acid binding protein 2 (HABP2), in vascular barrier regulation.

Methods and Results—By using immunoblot and immunohistochemical analysis, we observed that lipopolysaccharide (LPS) induces HABP2 expression in murine lung endothelium in vivo and in human pulmonary microvascular endothelial cells (ECs) in vitro. High-molecular-weight hyaluronan (HMW-HA, approximately 1×10⁹ Da) decreased HABP2 protein expression in human pulmonary microvascular ECs and decreased purified HABP2 enzymatic activity, whereas low-molecular-weight HA (LMW-HA, approximately 2500 Da) increased these activities. The effects of LMW-HA, but not HMW-HA, on HABP2 activity were inhibited with a peptide of the polyanion-binding domain of HABP2. Silencing (siRNA) HABP2 expression augmented HMW-HA–induced EC barrier enhancement and inhibited LPS and LMW-HA–mediated EC barrier disruption, results that were reversed with overexpression of HABP2. Silencing photosynthetically active radiation receptors 1 and 3, RhoA, or Rho kinase expression attenuated LPS-, LMW-HA–, and HABP2-mediated EC barrier disruption. By using murine models of acute lung injury, we observed that LPS- and ventilator-induced pulmonary vascular hyperpermeability was significantly reduced with vascular silencing (siRNA) of HABP2.

Conclusion—HABP2 negatively regulates vascular integrity via activation of photosynthetically active radiation receptor/RhoA/Rho kinase signaling and represents a potentially useful therapeutic target for syndromes of increased vascular permeability. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: HABP2 ■ hyaluronan ■ protease-activated receptor ■ endothelial barrier function ■ vascular permeability

Endothelial cells (ECs) constitute the inner lining of all blood vessels and regulate the interface between the circulating blood and the vessel wall, including vascular barrier regulation, passive diffusion, active transport of blood-borne substances, regulation of vascular smooth muscle tone, and blood coagulation. The disruption of this semiselective cellular barrier is a critical feature of inflammation and an important contributing factor to acute lung injury (ALI), an inflammatory condition that is a major cause of morbidity and mortality in critically ill patients because it results in leakage of fluid, protein, and cells into lung airspaces. Previous studies indicated that HMW-HA promotes EC barrier function, whereas low-molecular-weight hyaluronan (LMW-HA) causes EC barrier disruption. However, the exact mechanism by which HA regulates vascular permeability is incompletely defined. A potential target of HA is the extracellular protein called hyaluronic acid binding protein 2 (HABP2). The role of HABP2 in EC barrier regulation has not previously been described and is examined in the present study.

HABP2, also called factor VII–activating protease, is an extracellular serine protease involved in the extrinsic pathway of blood coagulation (via activation of factor VII) and fibrinolysis (via activation of prourokinase type plasminogen activator). HABP2 has been implicated in several disease processes, including atherosclerosis and deep venous thrombosis. HABP2, which is initially expressed in a single amino acid chain proenzymatic form, undergoes autocatalytic cleavage on binding of a ligand. Originally isolated based on its affinity for HA, HABP2 is capable of being activated by a variety of polyanions, including heparin and nucleic acids. The fully mature enzyme consists of trypsinlike catalytic domain, linked via disulfide bond to the kringle domain and 3 epidermal growth factor–like domains. The second and third epidermal growth factor–like domains form the polyanion-binding domain (PABD). HABP2 can form covalent complexes with certain inhibitors, including plasminogen activator inhibitor 1. Plasminogen activator inhibitor 1 is an important regulator of ALI. Interestingly, HABP2 expression and activity are upregulated in the lungs of patients with acute respiratory distress syndrome, a disease with prominent vascular leakiness. We examined the role of HABP2 in vascular integrity using in vitro models of pulmonary EC barrier function and in vivo models of ALI with pulmonary vascular hyperpermeability.

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Methods

Cell Culture and Reagents
Human pulmonary microvascular ECs were obtained from Cambrex, Walkersville, Md, and cultured as previously described4 in EBM-2 complete medium (Cambrex) at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air, with passages 6 to 10 used for experimentation. Unless otherwise specified, reagents were obtained from Sigma, St Louis, Mo. Reagents for sodium dodecyl–polyacrylamide gel electrophoresis were purchased from Bio-Rad, Richmond, Calif; Immobilon-P transfer membrane from Millipore Corp, Bedford, Mass; and gold microelectrodes from Applied Biophysics, Troy, NY. Mouse anti–HABP2 antibody was obtained from Novus Biologicals, Littleton, Colo. Mouse anti–photosynthetically active radiation (PAR) 1, anti–PAR-2, anti–PAR-3, anti–PAR-4, rabbit and goat anti–PAR blocking (N-terminal binding), and rabbit anti–Rho kinase (ROCK) antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, Calif. Mouse anti–RhoA antibody was obtained from Upstate Biotechnology, Lake Placid, NY. Rabbit anti–factor VIII antibody was purchased from Chemicon, Temecula, Calif. A synthetic peptide corresponding to the second and third epidermal growth factor–like repeats of human HABP2 was purchased from Abnova, Neihu District, Taipei City, Taiwan. Mouse anti–β-actin antibody, lipopolysaccharide (LPS), thrombin, and trypsin were purchased from Sigma. PPACK was purchased from EMD Chemicals, Gibbstown, NJ. Secondary horse-radish peroxidase–labeled antibodies were purchased from Amer sham Biosciences, Piscataway, NJ.

The preparation of LMW-HA and HMW-HA is the same as previously described.2

HABP2 Protein Purification and Protease Activity Determination
ECs were transfected with HABP2 overexpression vector (Origene, Rockville, Md), with Eugene HD (Roche, Indianapolis, Ind) as the transfection reagent for 72 hours. Media from the transfected ECs were immunoprecipitated with anti–HABP2 antibody (Novus Biologicals, Littleton, Colo) covalently linked to sepharose beads (AminoLink Plus Immobilization Kit; Pierce, Thermo Fisher Scientific, Rockford, Ill). The immunobeads were then extensively washed in 50-mmol/L borate, pH 8.5, and eluted with 1-mol/L sodium chloride with 0.1% NP-40 and dialyzed against a 1000-fold excess of 50-mmol/L borate, pH 8.5. Protein assays were performed to quantitate total protein. The purified protein was run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and either immunoblotted with anti–HABP2 antibody (Novus Biologicals) or stained with protein stain (Imperial; Pierce) to determine purity. Protease activity was measured using a commercially available kit (QuantiCleave Protease Assay Kit; Pierce). Briefly, the immunobeads are incubated with succinylated casein for various functional assays were conducted.

RNA Isolation
Total RNA was isolated from ECs and frozen lungs using a reagent (TRizol; Invitrogen, Carlsbad, Calif) and a kit (RNeasy; Qiagen, Valencia, Calif). Cells were lysed, and frozen lungs were homogenized in the reagent using a tissue homogenizer (Polyclone; Kine matica, Bohemia, NY). The kit (RNeasy) was used to further purify the isolated RNA. Each group consisted of either lungs from 6 animals or ECs from 3 wells of a 6-well plate.

Reverse Transcription–Polymerase Chain Reaction Analysis
Relative quantification was performed using real-time polymerase chain reaction (PCR) with a reverse transcription–PCR assay (TaqMan) and a PCR system (model 7900HT Fast Real-Time PCR System; Applied Biosystems, Foster City, Calif). The ready-made primer and probe sets were ordered from Applied Biosystems (catalog No. as follows: human HABP2, Hs00928793 m1; human GAPDH, Hs99999905 m1; mouse HABP2, Mm01254240 m1; and mouse GAPDH, Mm00475623 m1). Three replicates were run for each gene for each sample in a 384-well plate. Glyceraldehyde-3-phosphate dehydrogenase was used as the endogenous reference gene because it does not exhibit significant expression changes between samples.

Use of siRNA
The siRNA for PAR-1, PAR-2, PAR-3, PAR-4, RhoA, and ROCK was purchased from Santa Cruz Biotechnology. The siRNA for HABP2 was purchased from Origene. Human lung ECs were transfected with siRNA using a transfection reagent (siPORTamine; Ambion, Austin, Tex) according to the protocol provided by Ambion. Cells (approximately 40% confluent) were serum starved for 1 hour, and then incubated with siRNA for 6 hours in serum-free media. Serum-containing medium was then added (10% serum final concentration) for 42 hours before biochemical experiments and/or functional assays were conducted.

Measurement of EC Electric Resistance
ECs were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and transendothelial electric resistance measurements were performed using an electric cell substrate impedance sensing system obtained from Applied Biophysics, as previously described.14 The transendothelial electric resistance values from each microelectrode were pooled at discrete time points and plotted versus time as the mean±SE.

RhoA activity assays in human lung ECs were performed as described previously.15

Animal Preparation and Treatment With LPS
Male C57BL/6J mice (aged 8–10 weeks; Jackson Laboratories, Bar Harbor, Me) were anesthetized with intraperitoneal ketamine, 150 mg/kg, and acetylpromazine, 15 mg/kg, according to approved protocols. LPS, 2.5 mg/kg, or saline (control) was instilled intratracheally. The animals were allowed to recover for 24 hours; afterward, bronchoalveolar lavage total protein, polymorphonuclear leukocyte, and albumin analysis and/or lung immunohistochemistry was performed.3,16

Murine Lung Immunohistochemistry
To characterize protein expression in mouse lung vascular ECs, lung specimens from C57BL/6J control and LPS-treated mice were formalin fixed. 5-μm paraffin sections were obtained and hydrated, and epitope retrieval was performed (DakoCytomation Target Retrieval Solution; pH, 6.0; DakoCytomation, Carpinteria, Calif). The sections were then histologically evaluated by either 4− or 6−diamidino-2-phenylindole stain or anti-HABP2 or anti–factor VIII antibody and secondary fluorescent antibody (Alexa Fluor 610 and 350; Molecular Probes, Invitrogen). Negative controls for immunohistochemical analysis were used via the same method, previously described, but without primary antibody. Immunofluorescent-stained sections were photographed (×100) using an axioscope (Leica, Bannockburn, Ill).

Delivery of siRNA in Mice
Adult male C57BL/6J mice, aged 8 to 10 weeks, with an average weight of 20 to 25 g (Jackson Laboratories), were bred at a University of Chicago, Chicago, Ill, animal care center. All experimental protocols involving the use of animals were approved by the University of Chicago Institutional Animal Care and Use Committee for the humane treatment of experimental animals. HABP2 siSTABLE siRNA from Dharmacon, Lafayette, Colo, had the following sequence: 5′-AGACGGAGAUCCAGGUUUA-3′; and control siRNA (Luciferase): 5′-UAAGCUAUGAGGAGAUUAA-3′. HABP2 siRNA and control siRNA, 10 mg/kg, were injected into the jugular vein while the mice were under anesthesia. After 5 days of siRNA treatment, the mice were euthanized, and their lung and plasma specimens were collected.
Mechanical Ventilation of Mice

After 5 days of siRNA delivery in mice with siHABP2 or nsRNA at a dose of 10 mg/kg (see previous data), mice were anesthetized with an intraperitoneal injection of ketamine, 75 mg/kg, and acepromazine, 1.5 mg/kg. A tracheotomy was performed, and the trachea was cannulated with a 20-gauge 1-inch catheter (Penn-Century Inc, Philadelphia, Pa), which was tied into place to prevent an air leak. The animals were placed on a mechanical ventilator (Harvard Apparatus, Boston, Mass) for 4 hours with high tidal volume ventilation (40 mL/kg, 75 breaths per minute, and 0 positive end-expiratory pressure). After the experiment, the animals were euthanized by exsanguination while under anesthesia.

Results

Pulmonary Endothelial HABP2 Expression Is Regulated by LPS and HA

The role of the extracellular serine protease, HABP2, in vascular barrier regulation is unknown. Because HABP2 is upregulated in the plasma and lung tissue of patients with acute respiratory distress syndrome,11,13 we examined HABP2 expression levels in an inflammatory model of ALI with well-characterized vascular hyperpermeability and observed that HABP2 messenger RNA (Supplemental Figure 1) and protein (Figure 1A–C) levels are increased in the lungs and plasma of mice challenged with the intratracheal administration of LPS. Immunohistochemical analysis revealed that the murine pulmonary vasculature had a robust increase in HABP2 expression levels (Figure 1D). Similar to our in vivo results, we observed that LPS challenge of human pulmonary microvascular ECs in vitro increases expression of HABP2 protein (Figure 1E). We examined the effect(s) of HA, an EC barrier regulatory glycosaminoglycan, on HABP2 expression and observed the EC barrier disrupting form of HA; LMW-HA increased extracellular protein expression of HABP2, whereas the EC barrier enhancing high-MW form of HA (HWM-HA) caused a reduction in HABP2 expression (Figure 1E).

HA Regulates HABP2 Protease Activity

HABP2 was originally discovered through its ability to bind to HA.5 Therefore, we examined whether HA can regulate HABP2 protease activity in addition to protein expression. By using purified HABP2 protein isolated from the media of human pulmonary ECs overexpressing HABP2 (Supplemental Figure 2 and Supplemental Figure 3), we observed that LMW-HA increased extracellular protein expression of HABP2, whereas the EC barrier enhancing high-MW form of HA (HWM-HA) caused a reduction in HABP2 expression (Figure 2A).
dose-dependent manner (Figure 2B). The PABD of HABP2 has been implicated in regulating agonist-induced HABP2 protease activity. Our results indicate that the addition of a synthetic peptide of the PABD of HABP2 inhibits the LWM-HA, but not the HMW-HA, regulation of HABP2 activity, indicating that LWM-HA, but not HMW-HA, regulates HABP2 through interactions with the PABD (Figure 2C).

PAR Receptors Regulate HABP2-Mediated Endothelial Barrier Disruption

Next, we examined the role of HABP2 on EC barrier regulation in vitro and observed that overexpression of HABP2 in ECs augmented the barrier-disruptive effects of LPS and LMW-HA while inhibiting the barrier-enhancing effects of HMW-HA (Figure 3A). In contrast, silencing (siRNA) HABP2 expression in ECs inhibits the barrier-disruptive effects of LPS and LMW-HA while augmenting the barrier-enhancing effects of HMW-HA (Figure 3A). The addition of exogenous HABP2 directly induces EC barrier disruption in a dose-dependent manner (Figure 3B), with a pattern similar to that of another extracellular protease, thrombin (Figure 3B [inset]). Pretreatment of HABP2 with the thrombin inhibitor, PPACK, blocked EC barrier disruption (Supplemental Figure 4). Because thrombin exerts its cellular actions through PARs, we examined PAR receptor activation by silencing (siRNA) PAR expression in human pulmonary ECs and observed that exogenous addition of HABP2 to human ECs activates PAR-1, PAR-3, and PAR-4 (Figure 3C). We examined the functional significance of PAR activation by silencing (siRNA) PAR expression in human pulmonary ECs and observed that, similar to previously published reports, silencing PAR-1, PAR-3,
and/or PAR-4 attenuates thrombin-induced EC barrier disruption (Figure 3D). Interestingly, silencing PAR-1 and/or PAR-3 also inhibits HABP2-mediated EC barrier disruption (Figure 3D). In addition, PAR-1 and PAR-3 (but not PAR-2 or PAR-4) blocking antibody treatment of human ECs prevented HABP2-mediated EC barrier disruption (Figure 3E). These novel results indicate that HABP2 can regulate EC barrier function through a mechanism that involves PAR-1 and PAR-3 receptors.
HABP2-Mediated Endothelial Barrier Disruption Is Regulated by RhoA and ROCK

Thrombin-mediated EC barrier disruption is regulated by RhoA and ROCK activation.19 Given our results in Figure 3 that HABP2-induced EC barrier disruption is regulated by PAR receptors (similar to thrombin), we examined whether HABP2 can activate RhoA (formation of RhoA-GTP) in human ECs. Our results in Figure 4A indicate that HABP2 induces RhoA activation, which is attenuated by silencing (siRNA) PAR-1 or PAR-3 expression. Furthermore, silencing (siRNA) RhoA or ROCK significantly inhibits HABP2-mediated EC barrier disruption (Figure 4B).

HABP2 Is an Important Regulator of Vascular Permeability

Given our results indicating HABP2 can regulate EC barrier function in vitro, we next examined whether HABP2 can regulate vascular permeability in vivo. The inhibition of murine plasma and lung HABP2 expression using intravenous administration of siSTABLE HABP2 siRNA (Dharmacon) (Supplemental Figure 5) significantly attenuated pulmonary vascular hyperpermeability in LPS- and ventilator-induced models of ALI, as determined by bronchoalveolar lavage total protein, polymorphonuclear leukocyte, and albumin concentrations. These results indicate that HABP2 negatively regulates vascular integrity in vivo.

Discussion

In this study, we investigated the role of HABP2 in vascular barrier regulation. Our findings indicate that HABP2, although primarily localized in the plasma, is upregulated in the lung endothelium with LPS-induced ALI and in cultured human pulmonary ECs. The enzymatic activity of HABP2 is differentially regulated by HA, with HMW-HA inhibiting HABP2 protease activity and LMW-HA binding to the PABD of HABP2 and stimulating activity. Activated HABP2 induces protease-activated receptor signaling in ECs, which leads to activation of the actin regulatory molecules RhoA and ROCK and endothelial barrier disruption. The barrier-disruptive role of HABP2 is further confirmed by vascular silencing of HABP2 expression, which attenuated the vascular leakiness observed in LPS- and ventilator-induced lung injury.

In previous studies,2,3 it was demonstrated that HA is an important regulator of EC barrier function, with LMW-HA increasing permeability and HMW-HA attenuating vascular leakiness. Because HABP2 is known to interact with HA,5,22 we examined HA as a potential regulator of HABP2 activity and expression. Our results indicate that LMW-HA, similar to LPS, increases the expression of HABP2 in ECs. Interestingly, HMW-HA, unlike LPS or LMW-HA, attenuates HABP2 expression. The exact mechanisms of HA-mediated changes in HABP2 protein expression are currently being examined in our laboratory. Similar to HA’s diurnal effects on HABP2 expression, enzymatic activity assays demonstrate that LMW-HA increases and HMW-HA decreases HABP2 protease activity. Previous studies9,10 have shown that several negatively charged molecules, such as heparin and RNA, are capable of activating HABP2. Our study indicates that LMW-HA has a comparable effect; however, surprisingly, HMW-HA behaves in an opposite manner, inhibiting protease activity. The PABD of HABP2 regulates LMW-HA, but not HMW-HA, protease activity. This suggests that HMW-HA binds HABP2 at a site other than the PABD. The exact region of HMW-HA interaction with HABP2 remains unknown and is currently being studied in our laboratory.

Because HABP2 is a novel agent involved in EC barrier disruption, with a profile similar to thrombin, we investigated possible signaling mechanisms and observed that HABP2 activates PAR-1 and PAR-3, with consequent activation of the small GTPase, RhoA, and the serine/threonine kinase, ROCK. Interestingly, silencing PAR-4 expression inhibited thrombin, but not HABP2-mediated, EC barrier disruption, indicating that HABP2 and thrombin have similar, but not identical, modes of action. Although we did not observe PAR-2 regulation of HABP2.
Figure 5. Hyaluronic acid binding protein (HABP) 2 regulates vascular permeability in acute lung injury models in vivo. A through C, Male B6129N2 mice (aged 8–10 weeks) were treated intravenously with 10 mg/kg of in vivo stable scramble siRNA (control) or HABP2 siRNA (Dharmacon). After 4 days of siRNA treatment, mice were given lipopolysaccharide (LPS), 2.5 mg/kg, or saline (control) intratracheally. The animals were allowed to recover for 24 hours after LPS before bronchoalveolar lavage (BAL) fluid was collected and total protein (A), polymorphonuclear leukocyte (PMN) (B), and albumin (C) assays were determined, which is presented in graphical form (n=6 per condition). The asterisk represents a statistically significant (P<0.05) difference between control and LPS administration. There was also significant attenuation of LPS-induced vascular leakiness with HABP2 silencing (P<0.05). D through F, After 5 days of siRNA treatment, as described in part A, male B6129N2 mice (aged 8–10 weeks) were exposed to high tidal volume (40 mL/kg) for 4 hours, BAL fluid was collected, and total protein (D), PMN (E), and albumin (F) assays were determined (n=4 per condition). The asterisk represents a statistically significant (P<0.05) difference between spontaneously breathing (SB) and high tidal volume (VILI). There was also significant attenuation of VILI-induced vascular leakiness with HABP2 silencing (P<0.05, n=4 per group).
mediated EC barrier disruption in vitro, it is possible that PAR-2 can regulate HABP2 signaling in vivo because a target of HABP2, factor VIIa, in a complex with tissue factor can activate PAR-2. HABP2 has been implicated in several disease processes, including atherosclerosis, ALI/acute respiratory distress syndrome, deep venous thrombosis, and cancer. In addition, the G534E mutation of HABP2 is associated with cardiovascular disease and thromboembolism.26–28 However, the mechanism by which HABP2 contributes to disease processes remains elusive. In normal blood coagulation cascade regulation, the role of HABP2 is complex because it activates factors involved in both coagulation and fibrinolysis.6 Our data that HABP2 negatively regulates EC barrier function provide important mechanistic insights into HABP2 involvement in diseases associated with vascular dysfunction. Furthermore, HABP2 regulation of a pulmonary vascular leak with ALI suggests a potential link between lung dysfunction and blood coagulation components. This is supported by reports that indicate changes in plasma levels of coagulation/fibrinolysis regulators (thrombomodulin, protein C, and plasminogen activator inhibitor 1) are associated with mortality in patients with ALI.

In summary, we have demonstrated that HABP2 increases vascular permeability through activation of a PAR receptor/RhoA/ROCK signaling pathway. Our data indicate that HABP2 is a contributing factor to the vascular leakiness associated with ALI. Identifying potential agents that can specifically inhibit HABP2 will have significant clinical utility in treating diseases involving defects in vascular permeability.

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Disclosures

None.

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Supplement Figure I – Analysis of HABP2 mRNA levels. Panel 1: HABP2 mRNA was quantitated from the lungs of control or LPS-treated mice as described in Methods. Panels 2-4: Total RNA was extracted from EC and analyzed for HABP2 mRNA expression as described in Methods. The fold change of HABP2 mRNA expression with LPS (Panel 2), HMW-HA (Panel 3) and LMW-HA (Panel 4) is graphically depicted.
Supplement Figure II – Analysis of the molecular weight and purity of HABP2. Panel 1 – Human endothelial cell (EC) lysate, mouse plasma and lung homogenates were immunoblotted for HABP2 using an antibody against the PABD of HABP2. The major immunoreactive band is at ~50 kDa. Panel 2 – HABP2 positive control (HABP2 overexpressing HEK293 cell lysate from Origene) and purified HABP (see Methods) were immunoblotted for HABP2 using an antibody against the PABD of HABP2. Two major bands at ~75 and 50 kDa are present indicating the pro- and active form of HABP2. Purified HABP2 was also Coomassie stained (Imperial™ protein stain (Pierce)) to demonstrate purity.
Supplement Figure III – Determination of the transfectional efficiency of human pulmonary microvascular EC. Various concentrations of pCMV6-AC plasmid overexpressing GFP (Origene) were transfected into EC using 10 ml Fugene HD transfection reagent (Roche) in 10 ml media (final volume). Transfected EC were grown for 48 hours and % transfection was quantitated using GFP fluorescence per cell. The asterisk indicates the maximal transfectional efficiency (~45%) and this condition was used to overexpress HABP2 with the pCMV6-AC plasmid (Origene) in EC as described in the manuscript. Panel 2: EC were either untransfected, transfected with HABP2 siRNA or an HABP2 overexpression pCMV6-AC vector (Origene) for 48 hours. Then, media was collected, concentrated, run on SDS-PAGE and immunoblotted with anti-HABP2 antibody.
Supplement Figure IV – Quantitation of HABP2-mediated human EC barrier disruption. Graphical depiction of % decrease in purified HABP2-mediated (with or without pre-incubation with 1 μM PPACK) EC resistance from three different experiments performed on three different days.
Supplement Figure V – Analysis of siRNA inhibition of protein expression. Panel 1: EC were either untransfected (control) or transfected with scramble siRNA, PAR-1 siRNA, PAR-2 siRNA, PAR-3 siRNA or PAR-4 siRNA for 48 hours. EC lysates were collected, run on SDS-PAGE and immunoblotted with anti-PAR-1 (a), anti-PAR-2 (b), anti-PAR-3 (c), anti-PAR-4 (d) or anti-actin (e) antibody. Panel 2: EC were either untransfected (control) or transfected with scramble siRNA, RhoA siRNA or ROCK siRNA for 48 hours. EC lysates were collected, run on SDS-PAGE and immunoblotted with anti-RhoA (a), anti-ROCK (b) or anti-actin (c) antibody. Panel 3: Male B6129N2 mice (8-10 weeks) were treated i.v. with 10 mg/kg in vivo stable scramble siRNA (control) or HABP2 siRNA (Dharmacon) for 5 days and plasma and lungs were collected. Extracted lungs were homogenized and plasma prepared, N=3 samples per condition were pooled, run on SDS-PAGE and immunoblotted with anti-HABP2 (a,c) or anti-actin (b) antibody.