Vascular Pharmacology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

The minor histocompatibility antigen 1 (HMHA1)/ArhGAP45 is a RacGAP and a novel regulator of endothelial integrity

J. Amado-Azevedo[a], N.R. Reinhard[b], J. van Bezu[a], G.P. van Nieuw Amerongen[a], V.W.M. van Hinsbergh[a], P.L. Hordijk[a,*]

[a] Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands
[b] Department of Molecular Cytology, Swammerdam Institute for Life Sciences, van Leeuwenhoek Centre for Advanced Microscopy, University of Amsterdam, Amsterdam, The Netherlands

ARTICLE INFO

Keywords:
HMHA1
ArhGAP45
Endothelium
Rac1
Permeability

ABSTRACT

Endothelial cells line the vasculature and act as gatekeepers that control the passage of plasma, macromolecules and cells from the circulation to the interstitial space. Dysfunction of the endothelial barrier can lead to uncontrolled leak or edema. Vascular leakage is a hallmark of a range of diseases and despite its large impact no specialized therapies are available to prevent or reduce it. RhoGTPases are known key regulators of cellular behavior that are directly involved in the regulation of the endothelial barrier. We recently performed a comprehensive analysis of the effect of all RhoGTPases and their regulators on basal endothelial integrity. In addition to novel positive regulators of endothelial barrier function, we also identified novel negative regulators, of which the ArhGAP45 (also known as HMHA1) was the most significant. We now demonstrate that ArhGAP45 acts as a Rac-GAP (GTPase-Activating Protein) in endothelial cells, which explains its negative effect on endothelial barrier function. Silencing ArhGAP45 not only promotes basal endothelial barrier function, but also increases cellular surface area and induces sprout formation in a 3D-fibrin matrix. Our data further shows that loss of ArhGAP45 promotes migration and shear stress adaptation. In conclusion, we identify ArhGAP45 (HMHA1) as a novel regulator, which contributes to the fine-tuning of the regulation of basal endothelial integrity.

1. Introduction

Endothelial cells line all blood vessels and execute various homeostatic functions. One of the main functions of the endothelium is, besides controlling gas exchange, forming a physical barrier that not only separates the blood and its components from the surrounding tissues but also tightly controls the passage of water, electrolytes, proteins and leukocytes. Dysfunction of the endothelial barrier leads to plasma- and leukocyte extravasation which correlates with life-threatening conditions such as sepsis [1], acute lung injury [2], anaphylaxis, cancer and diabetic complications [3,4]. Maintenance of the endothelial barrier is controlled by various mechanical, hormonal, vasoactive and metabolic factors mainly at the level of intercellular adherens junctions. These adherens junctions are formed by protein-protein interactions between transmembrane adhesion molecules such as vascular endothelial (VE)-cadherin. VE-cadherin is regulated by the dynamic, contractile function of the associated F-actin cytoskeleton.

RhoGTPases are key regulators of cytoskeletal dynamics, impinging on many aspects of cell behavior such as polarity, adhesion and migration. RhoGTPases regulate both basal and stimulus-controlled integrity of the endothelial barrier in positive and negative ways [5–8]. We recently showed that a Cdc42/Rac1-centered signaling unit is a major positive regulator of basal barrier function [9], which counteracts a RhoB-dependent pathway and promotes contractility and endothelial permeability in resting endothelium [10]. The activation of and localized signaling by these GTPases is controlled by groups of regulatory proteins which are in turn activated through cell-surface receptors and adhesion molecules. So far, three classes of regulatory proteins have been described: GEFs (Guanine nucleotide Exchange Factors) that induce RhoGTPase activation by promoting exchange of bound GDP for GTP (the 'on' state); GAPs (GTPase Activating Proteins) that promote hydrolysis of GTP to GDP, switching the GTPase to its inactive, 'off', state; and GDIs (Guanine nucleotide Dissociation Inhibitors), cytosolic chaperones which maintain GTPases in their inactive state. Once activated, GTPases transmit their signal to downstream effector proteins to control myosin contractility or F-actin polymerization.

To date, there are 22 RhoGTPases described, > 80 GEFs, ~60 GAPs

* Corresponding author.
E-mail address: p.hordijk@vumc.nl (P.L. Hordijk).

https://doi.org/10.1016/j.vph.2017.11.007
Received 13 September 2017; Received in revised form 14 November 2017; Accepted 18 November 2017

and 3 GDIs [11–14]. Yet, knowledge on localized (in)activation and mechanisms of regulation is limited to a few GEF/GAP family members, particularly those regulating the best studied GTPases: RhoA, Rac1, Cdc42 and Rap1. Recently, we identified, through a systematic siRNA screen in primary human endothelium, a cdc42 centered signaling unit as a positive regulator of endothelial barrier function [9], while the RhoGAP ArhGAP45 appeared an important player in the negative regulation of basal endothelial barrier function. ArhGAP45 is a newly identified RhoGAP that has been shown to interact directly with RhoA and Rac1 [15]. Sequence analysis of ArhGAP45 shows its similarity to other known GAPs such as GRAF1 and p50RhoGAP [15]. Furthermore, ArhGAP45 was also found to encode a N-terminal BAR domain. BAR domains are involved in membrane dynamics (especially in sensing and inducing membrane curvature), as well as in vesicle transport and regulation of GTPase activity and function [16]. One sub-class of BAR-domain proteins, including SH3BP1, OPHN1 and GRAF1 have a very similar structure to ArhGAP45 and all of them include in their sequence both a BAR- and GAP-domain [17–19].

ArhGAP45 is also known as Human Minor Histocompatibility Antigen 1 (HMHA1). Human minor histocompatibility antigens were initially detected following organ graft rejection which stresses their role in immunological barriers. These antigens are also the target of immune responses after allogeneic stem cell transplantation used to treat a variety of malignancies, such as leukemia or solid tumors [20]. The HMHA1 gene encodes the minor histocompatibility antigen-1 (HA-1) which is directly correlated with hematological malignancies, namely in acute myeloid leukemia.

Here we identify ArhGAP45 (HMHA1) as a novel regulator of the endothelial barrier through a systematic, loss-of-function siRNA screen. HUVECs lacking ArhGAP45 show a significant increase in basal barrier function and cell migration. FRET biosensor analysis in HUVECs shows that ArhGAP45 interacts preferentially with and regulates Rac1 as compared to RhoA or Cdc42. Under conditions of shear stress, endothelial cells lacking ArhGAP45 align faster and migrate more efficiently.

2. Material and methods

2.1. Reagents and antibodies

For protein analysis (western blot) we used: anti-HMHA1 (Sigma Aldrich, St Louis, MO); anti-VE-cadherin (Cell Signaling Technologies, Danvers, MA); anti-β-Tubulin (Cell Signaling Technologies, Danvers, MA) and anti-ERK 2 (Santa Cruz Biotechnology, Inc. Dallas, TX). Secondary antibodies were from Invitrogen, Paisly, UK. For additional stainings, we used DAPI for nuclei (Thermo Fisher Scientific) and Rhodamine-Phalloidin for F-actin (Invitrogen Corporation, San Diego, CA), anti-VE-cadherin XP (Cell Signaling Technologies, Danvers, MA) and anti-Vinculin (Sigma Aldrich, St Louis, MO). FITC-labeled- and Alexa 647-linked secondary antibodies were from (Thermo Fisher Scientific, Waltham, MA). For other assays, the following reagents were used: Fibronectin (Stago bnl, Leiden, The Netherlands); Thrombin (Sigma Aldrich, Zwijndrecht, The Netherlands); Tumor Necrosis Factor-α (TNFα) (Sigma, St Louis, USA); Fibroblast Growth Factor-2 (FGF2) (Preprotech, London, UK); Paraformaldehyde (Merck, Darmstadt, Germany); Human Serum Albumin (Sanquin, Amsterdam, The Netherlands); Histamine (Tocris Bioscience, Ellisville, MO), Sphingosine-1-phosphate (S1P) and Nocodazole (both from Sigma Aldrich, St Louis, MO).

2.2. Endothelial cell culture

Human umbilical vein endothelial cells were freshly isolated from umbilical cords of healthy donors, as previously described [21] and were obtained at the Amstelland Ziekenhuis (Amstelveen, The Netherlands). Informed consent was obtained from all donors in accordance with the institutional guidelines and the Declaration of Helsinki. After isolation, cells of different donors were pooled and resuspended in M199 medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mmol/l L-glutamine (all Lonza, Belgium), 10% heat-inactivated human serum (Invitrogen, WI, USA), 10% heat-inactivated new-born calf serum (Lonza, Belgium), 150 μg/ml crude endothelial cell growth factor (prepared from bovine brains), 5 U/ml heparin (Leo Pharmaceutical Products, Breda, The Netherlands) and seeded on 1% gelatin-coated plates. Cells were cultured at 37 °C and 5%CO_2 with a change of medium every other day. For all experiments, pools of HUVECs of at least 3 donors in passage 2 were used.

FRET measurements: Primary HUVEC were obtained from Lonza and cultured on fibronectin-coated dishes in EGM-2 medium, supplemented with singlequots (Lonza, Verviers, Belgium). Cells were cultured at 37 °C and 5%CO_2 with a change of medium every other day.

2.2.1. hMVECs

Human microvascular endothelial cells were isolated from foreskin, kindly provided by the Department of Dermatology (VU University Medical Center, Amsterdam, The Netherlands), cultured and characterized as previously described [22]. Informed consent was obtained from all donors in accordance with the institutional guidelines and the Declaration of Helsinki. hMVECs were cultured on 1% gelatin coated culture plates in culture medium that consisted of M199, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mmol/l L-glutamine (all Lonza, Belgium), 10% heat-inactivated human serum (Invitrogen, WI, USA), 10% heat-inactivated new-born calf serum (Lonza, Belgium), 150 μg/ml crude endothelial cell growth factor (prepared from bovine brains), 5 U/ml heparin (Leo Pharmaceutical Products, Breda, The Netherlands). Cells were cultured at 37 °C and 5%CO_2 with a change of medium every other day. For experiments, hMVECs of individual donors up to passage 10 were used.

2.2.2. HAECs

Human aortic endothelial cells were a kind gift of Dr. P. Koolwijk, VU University Medical Center, Amsterdam, The Netherlands. Informed consent was obtained from all donors in accordance with the institutional guidelines and the Declaration of Helsinki. These cells were isolated and characterized as previously described [23]. HAECs were cultured on 1% gelatin-coated culture plates in EGM2 medium (Lonza, Walkersville, MD) supplemented with 10% platelet lysate [24]. Cells were cultured at 37 °C and 5%CO_2 with a change of medium every other day. For experiments, HAECs of individual donors up to passage 5 were used.

2.2.3. ECFCs

Peripheral blood-derived endothelial colony-forming cells (ECFCs) were a kind gift from D. Tasev, VU University Medical Center, Amsterdam, The Netherlands. Informed consent was obtained from all donors in accordance with the institutional guidelines and the Declaration of Helsinki. These cells were isolated, expanded and characterized as previously described [24]. After positive flow-cytometry immuno-characterization, cells were expanded for 1–2 passages in complete medium that contained M199, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mmol/l L-glutamine (all Lonza, Belgium), 10% heat-inactivated human serum (Invitrogen, WI, USA), 10% heat-inactivated new-born calf serum (Lonza, Belgium), 150 μg/ml crude endothelial cell growth factor (prepared from bovine brains), 5 U/ml heparin (Leo Pharmaceutical Products, Breda, The Netherlands).

2.3. siRNA transfections

Forward transfections were performed according to manufacturer's instructions and using ON-TARGETplus siRNA pools (ArhGAP45 – L-023893-00-0005; Non-targeting D-001810-10-05) at 25 nM final concentration and 0.25%(v/v) of Dharmafect 1 transfection reagent