Riociguat inhibits ultra-large VWF string formation on pulmonary artery endothelial cells from chronic thromboembolic pulmonary hypertension patients

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Abstract
Chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by elevated pulmonary arterial pressure and organized thrombi within pulmonary arteries. Riociguat is a soluble guanylate cyclase stimulator and is approved for patients with inoperable CTEPH or residual pulmonary hypertension after pulmonary endarterectomy (PEA). Previous work suggested that riociguat treatment is associated with an increased risk of bleeding, although the mechanism is unclear. The aim of this study is to assess how riociguat affects primary hemostasis by studying its effect on the interaction between platelets and endothelial cells derived from CTEPH patients. Pulmonary artery endothelial cells (PAECs) were isolated from thrombus-free regions of PEA material. Purified PAECs were cultured in flow chambers and were stimulated with 0.1 and 1 µM riociguat for 24 h before flow experiments. After stimulation with histamine, PAECs were exposed to platelets under shear stress. Platelet adhesion and expression of von Willebrand Factor (VWF) were evaluated to assess the role of riociguat in hemostasis. Under dynamic conditions, 0.1 and 1.0 µM of riociguat suppressed platelet adhesion on the surface of PAECs. Although riociguat did not affect intracellular expression and secretion of VWF, PAECs stimulated with riociguat produced fewer VWF strings than unstimulated PAECs. Flow cytometry suggested that decreased VWF string formation upon riociguat treatment may be associated with suppressed cell surface expression of P-selectin, a protein that stabilizes VWF anchoring on the endothelial surface. In conclusion, Riociguat inhibits VWF string elongation and platelet adhesion on the surface of CTEPH-PAECs, possibly by reduced P-selectin cell surface expression.
INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is diagnosed when despite effective anticoagulation treatment, persistent thromboembolic obstruction of pulmonary arteries leads to an elevated mean pulmonary arterial pressure. Patients left untreated may die from right heart failure. Treatment options for CTEPH are pulmonary endarterectomy (PEA), balloon pulmonary angioplasty (BPA), and medical therapies, which together significantly improve the prognosis of CTEPH. PEA is the first choice of treatment and involves removal of organized thrombi from the pulmonary arteries. Nevertheless, 16%–50% of patients who underwent PEA suffer from residual or recurrent pulmonary hypertension (PH). In addition, according to an international registry, 30%–40% of CTEPH patients are inoperable due to surgical inaccessibility, compromised hemodynamics, and/or comorbidities. Medical treatments with specific pulmonary vasodilators serve as alternatives to patients with inoperable CTEPH or residual PH after PEA and BPA. Riociguat was the first medical therapy specifically approved for CTEPH patients. This selective pulmonary vasodilator acts on the nitrergic pathway and reduces pulmonary vasconstriction by stimulating soluble guanylate cyclase (sGC). Clinically, it has been shown that riociguat improved exercise capacity, hemodynamics and cardiac function in CTEPH patients in the short and longer term. Life-long anticoagulation is required in all CTEPH patients to prevent de novo pulmonary embolism. However, the combined use of selective pulmonary vasodilators and anticoagulants seems to be associated with an increased risk of bleeding. In our previous study, riociguat treatment increased the bleeding risk in CTEPH patients treated with vitamin K antagonists. However, the mechanism by which vasodilators elicit bleeding remains enigmatic.

Vascular thrombosis is initiated when platelets bind to von Willebrand Factor (VWF) secreted from endothelial cells (ECs). Under physiological flow, endothelial cells have a negative charge and release prostaglandin I_2 and nitric oxide to prevent platelet adhesion. Activation of ECs by, for example, a stress response or inflammation leads to the release of VWF from Weibel-Palade bodies (WPB), and the formation of ultra-large VWF (ULVWF) strings, which serves as a platform for platelets to adhere to initiate coagulation. VWF strings are anchored and stabilized on the endothelial surface by P-Selectin and α_5β_3 integrin. We have recently demonstrated that VWF-mediated platelet adhesion is increased in PAEC-derived CTEPH patients. However, the effect of riociguat on these cells has never been investigated. It has been known that riociguat can suppress activation and aggregation of platelets via suppression of glycoprotein IIb/IIIa, but the effect on endothelial cells is unclear. We hypothesized that riociguat would affect ULVWF string formation and platelet adhesion, thereby possibly contributing to bleeding.

The aim of this study was to evaluate the effect of riociguat on VWF and endothelium-platelet interactions under dynamic conditions. We found that platelet adhesion on the CTEPH endothelium was inhibited by riociguat, which was associated with decreased VWF string formation and a trend to reduced endothelial surface expression of P-selectin.

METHODS

Reagents

Riociguat was purchased from Sigma Aldrich, dissolved in dimethyl sulfoxide (DMSO) in a concentration of 1 mol/L and stored at -80°C. Histamine was purchased from Sigma-Aldrich, dissolved in supplemental free endothelial cell medium in a concentration of 1 M and stored ad -20°C. Antibodies against the following proteins were used: VWF (1:1000, A0082; Dako), β-actin (1:1000, SC-47778; Santa Cruz Biotechnology). Primary antibodies were detected with secondary antibodies for polyclonal goat anti-rabbit (1:2500, P0448; Dako) or anti-mouse antibodies (1:2500, P0449; Dako) conjugated with horseradish peroxidase (HRP).

Isolation and culture of pulmonary arterial endothelial cells

PAECs were isolated and purified according to the previously published protocol. Briefly, chronic thrombi were surgically resected from pulmonary endarterectomy material obtained from CTEPH patients. The endothelial inner layer was scratched with a scalpel, transplanted onto a 60 mm culture dish (Corning) coated with 5 μg/ml fibronectin, and incubated with complete endothelial cell medium (cECM; ScienCell Research...
Laboratories) at 37°C and 5% CO₂ in a humidified incubator. For purification, the outgrown endothelial cells were separated with CD144 positive magnetic beads (Miltenyi Biotec). Purified cells were expanded on 0.1% gelatin until passage 4-6 was reached for the following experiments.

**Flow experiment**

PAECs were cultured on µ-Slide VI 0.4 ibidi flow chambers (ibidi GmbH) coated with 0.1% gelatin until confluence. Before the flow experiments, PAECs were stimulated with 0.1 and 1 µM riociguat for 24 h. After stimulation, PAECs were additionally stimulated with 1 µM histamine for 5 min to induce VWF secretion.

Platelets were freshly collected and prepared on the day of the experiments. Citrated blood was centrifuged at 150g and platelet-rich plasma (PRP) was collected in a new tube. Ten percent of acid citrate dextrose (ACD 85 mM sodium citrate, 65 mM citric acid, 100 mM glucose) was added for anticoagulation and PRP was centrifuged at 2000g. The supernatant was removed and platelets were fluorescently stained with Calcein AM (1:1000; Thermo Fisher Scientific) for 15 min. Platelets were fluorescently stained with Calcein AM (1:1000; Thermo Fisher Scientific) for 15 min. Platelets were washed with platelet wash buffer (36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 56 mM Tween 20 (TBS-T, pH 7.6) and overnight incubated at 4°C. The wells were washed with PBS containing anti-VWF antibody (1:1000, A0082; Dako) and incubated overnight at 4°C with primary antibodies. Secondary antibodies were incubated for 1 h at room temperature. Protein bands were detected using 5% BSA in Tris-buffered saline containing Tween-20 (TBS-T, pH 7.6) and overnight incubated at 4°C with primary antibodies. Secondary antibodies were incubated for 1 h at room temperature. Protein bands were detected using ECL Prime Blotting Detection Reagent (GE Healthcare Life Sciences) and imaged by Amersham™ Imager 600 (GE Healthcare Life Sciences). Bands were quantified with ImageJ and normalized to loading control.

**Real-time quantitative polymerase chain reaction (qPCR)**

Total RNA of PAEC stimulated with 0.1 or 1.0 µM riociguat with 0.1 or 1.0 µM riociguat for 24 h using lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 100 mM KCl, 2 mM EDTA-NaOH, 5% Igepal, and 0.5% Triton-X) supplemented with phosphatase and protease inhibitor cocktail (Roche). Western blot analysis was performed according to our previously published report. In brief, lysates were prepared with 1× NuPage LDS sample buffer (Thermofisher Scientific) and 50 µM DTT (Thermofisher Scientific). Protein samples were separated on 4%–12% NuPageTM Bis-Tris protein gel (Thermofisher Scientific) and transferred to 0.45 µm nitrocellulose membranes (Thermofisher Scientific). Protein membranes were blocked with 5% BSA in Tris-buffered saline containing Tween-20 (TBS-T, pH 7.6) and overnight incubated at 4°C with primary antibodies. Secondary antibodies were incubated for 1 h at room temperature. Protein bands were detected using ECL Prime Blotting Detection Reagent (GE Healthcare Life Sciences) and imaged by Amersham™ Imager 600 (GE Healthcare Life Sciences). Bands were quantified with ImageJ and normalized to loading control.

**Protein isolation and western blot analysis**

Total protein lysates were collected from PAECs stimulated with 0.1 or 1.0 µM riociguat for 24 h, using lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 100 mM KCl, 2 mM EDTA-NaOH, 5% Igepal, and 0.5% Triton-X) supplemented with phosphatase and protease inhibitor cocktail (Roche). Western blot analysis was performed according to our previously published report. In brief, lysates were prepared with 1× NuPage LDS sample buffer (Thermofisher Scientific) and 50 µM DTT (Thermofisher Scientific). Protein samples were separated on 4%–12% NuPageTM Bis-Tris protein gel (Thermofisher Scientific) and transferred to 0.45 µm nitrocellulose membranes (Thermofisher Scientific). Protein membranes were blocked with 5% BSA in Tris-buffered saline containing Tween-20 (TBS-T, pH 7.6) and overnight incubated at 4°C with primary antibodies. Secondary antibodies were incubated for 1 h at room temperature. Protein bands were detected using ECL Prime Blotting Detection Reagent (GE Healthcare Life Sciences) and imaged by Amersham™ Imager 600 (GE Healthcare Life Sciences). Bands were quantified with ImageJ and normalized to loading control.

**Enzyme-linked immunosorbent assay (ELISA)**

Supernatant was collected from PAECs stimulated with 0.1 or 1.0 µM riociguat for 24 h and secreted VWF levels were measured as previously described. In brief, phosphate-buffered saline (PBS) containing anti-VWF antibody (1:1000, A0082; Dako) was coated into each well of a 96-well plate and incubated overnight at 4°C. The wells were washed with PBS and blocked using PBS containing 2% BSA at room temperature for 2 h. After blocking, supernatants were applied into each well and incubated at room temperature for 2 h. After washing, HRP-conjugated rabbit polyclonal anti-VWF was added at room temperature for 2 h. VWF levels were detected using substrate solution and the reaction was stopped by a stop solution 20 min later. Absorbances of 450 and 540 nm were read using a
plate reader. The VWF concentration was determined by a standard curve.

**Immunofluorescence imaging of secreted VWF**

PAECs were cultured on μ-Slide VI 0.4 ibidi flow chambers coated with 0.1% gelatin until confluence. PAECs were stimulated with 0.1 or 1.0 μM riociguat for 24 h and additionally stimulated with histamine to induce VWF secretion. VWF elongation was induced by applying shear stress of 2.5 dyne/cm² for 5 min, after which PAECs were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). Cells were blocked with 1% BSA for 30 min at RT, and stained with fluorescein isothiocyanate conjugated VWF (1:1000), Phalloidin (1:400; Cytoskeleton), and Hoechst (1:1000; Thermofisher Scientific) for 30 min at RT. Confocal images were acquired with Nikon A1R and Z-stacks of 2 μm were taken with a ×60 oil-immersion objective.

**Flow cytometry**

PAECs were pretreated with riociguat and stimulated with histamine to evaluate P-selectin surface expression. Cells were detached, blocked for 10 min with 10% BSA, and stained for P-selectin (1:50, CD62P, 1E3, sc-19672) for 1 h at 4°C. Fluorescently labelled secondary antibody was incubated for 30 min at 4°C and signals were measured using Attune™ NxT Flow Cytometer. Unstained and secondary antibody stained were used as negative controls. The mean fluorescent intensity (MFI) of P-selectin was analyzed with FCS Express version 7.

**Statistical analysis**

Continuous variables were described as mean ± standard deviation (SD) unless otherwise stated. All analyses were performed using GraphPad prism ver.9.0 (GraphPad Software Inc.). Comparison between two groups with normal distribution was performed using an unpaired Student's t test. If not normally distributed, a Mann–Whitney U test was used. For comparison with more than two groups, two-way analysis of variance with Greenhouse-Geisser correction was used, followed by Tukey’s post hoc for multiple comparisons. p < 0.05 was considered significant.

**RESULTS**

**Riociguat inhibits platelet adhesion on CTEPH pulmonary artery endothelium**

PAECs were isolated from the surgical specimens of 12 CTEPH patients (Table 1). The baseline hemodynamic characteristics of CTEPH patients are displayed in Supporting Information: Table S1. To assess the in vitro effect of riociguat on platelet adhesion to CTEPH endothelium, freshly isolated platelets were perfused over PAEC monolayers which were incubated with riociguat for 24 h, followed by histamine stimulation for 30 min (Figure 1a). The area covered by platelets after histamine stimulation dropped by 60% on PAECs pretreated with riociguat (15.94 ± 1.19% vs. 6.15 ± 3.38%) (Figure 1b), with a comparable decrease observed for 0.1 and 1.0 μM. These data indicate that riociguat suppresses platelet adhesion.

**Riociguat did not affect VWF intracellular expression and secretion in CTEPH-PAEC**

We have recently shown that platelet adhesion on CTEPH endothelium is driven by excessive VWF expression. To examine the effect of riociguat on VWF expression in CTEPH-PAEC, analysis on RNA and protein expression was performed. Stimulation with riociguat did not alter VWF messenger RNA (mRNA) expression (Figure 2a) or the levels of intracellular VWF protein in CTEPH PAECs (Figure 2b,c). Since platelets bind to secreted VWF, we next evaluated the effect of riociguat on VWF release upon histamine stimulation. However, no changes in VWF release were observed after riociguat pretreatment (Figure 2d). Together, these data show that riociguat did not suppress intracellular VWF expression or release from CTEPH-PAEC in static conditions.
**FIGURE 1**  Riociguat inhibits platelet adhesion on CTEPH pulmonary artery endothelium (a) Brightfield and fluorescence images of adhered platelets (green) on Riociguat pretreated PAEC monolayers stimulated with or without histamine. (b) Comparison of total adhered platelets by quantifying the area covered by platelets (n = 5). Significance is indicated with ****p < 0.0001 after two-way ANOVA with Greenhouse-Geisser correction for Tukey’s comparison test. ANOVA, analysis of variance; CTEPH, chronic thromboembolic pulmonary hypertension; DMSO, dimethyl sulfoxide; FOV, fields of view.

**FIGURE 2**  Riociguat did not affect VWF expression and secretion in CTEPH-PAEC. CTEPH-PAECs were pretreated with riociguat and total cell lysates were used to determine (a) VWF mRNA expression (n = 5) and (b, c) total protein expression (n = 4). Data was not significant. (d) Endothelial VWF release from PAEC with or without histamine activation (n = 4). Significance is indicated with *p < 0.05 and **p < 0.01 after two-way ANOVA with Greenhouse-Geisser correction for Tukey’s comparison test. ANOVA, analysis of variance; CTEPH, chronic thromboembolic pulmonary hypertension; DMSO, dimethyl sulfoxide; mRNA, messenger RNA; VWF, von Willebrand factor.
VWF string formation under shear was inhibited by riociguat

Under laminar flow, confluent endothelial monolayers release VWF that form strings parallel to the direction of flow. These extend to several hundreds of micrometers and are stabilized on the surface of the endothelium. Alterations in VWF strings may disturb hemostasis. To evaluate whether riociguat interfered with this mechanism, VWF string formation under shear was studied after immunofluorescence visualization (Figure 3a). Upon shear, riociguat treatment significantly reduced the number of VWF strings (Figure 3b) and individual VWF strings tended to be shorter after riociguat treatment of PAEC, although the difference was not statistically significant (Figure 3c). The total string length of VWF was significantly lower (Figure 3d). This effect may lead to decreased stabilization of platelet binding under flow.

**FIGURE 3**

VWF string formation under shear was inhibited by riociguat (a) Immunofluorescence images of extracellular VWF string formation (green) under shear on histamine activated, and riociguat pretreated PAEC. Shear stress was applied for 5 min and endothelial cells were identified with Phalloidin (red), nuclei were stained with Hoechst (blue). Scale bar = 50 μm. (b) Quantification of total number of strings (c) Quantification of single string length (d) Quantification of total VWF length. Significance is indicated with *p < 0.05, **p < 0.01 after Kruskal–Wallis test with Dunn’s multiple comparison test. CTEPH, chronic thromboembolic pulmonary hypertension; VWF, von Willebrand factor.
Riociguat inhibits P-selectin surface expression

To explain our observation of fewer and shorter VWF strings, we considered that some factors associated with VWF stabilization under shear stress were affected by riociguat. It has been reported that P-selectin and α_vβ_3 integrin are involved in the stabilization of VWF on the cell surface.19,20 Thus, the effects of riociguat on the expression of these adhesion molecules in CTEPH-ECs were assessed. Total P-selectin and α_vβ_3 protein expression were not affected by riociguat (Figure 4a,b, Supporting
Information: Figure S1A-B). However, because VWF anchoring depends on the cell surface expression of these adhesion molecules, we performed flow cytometry analysis (Figure 4c). Stained and unstained cells were mixed to determine the range of positive signals (Figure 4d), which was used to quantify cell surface expression.

Pretreatment with riociguat did not affect the percentage of P-selectin-positive cells. P-selectin surface expression was low under basal conditions but increased after stimulation with histamine for 30 min (Figure 4e). The histamine-induced surface expression of P-selectin was abrogated by riociguat, independent of concentration (Figure 4f). However, because of variability between samples, we observed only a trend to a lower histamine-related induction of P-selectin on the cell surface after riociguat treatment (Figure 4g). Riociguat treatment did not affect αvβ3 integrin expression (Supporting Information: Figure S1). Although the effect of riociguat on P-selectin was modest, these data suggest that P-selectin may be involved in riociguat-mediated suppression of platelet adhesion on activated PAEC.

**DISCUSSION**

As riociguat has been reported to increase the risk of bleeding as a side effect, the present study aimed to understand how riociguat affects primary hemostasis. Investigating the effect of riociguat on platelet adhesion to CTEPH PAECs, we found that riociguat reduced VWF strings formation on PAEC contributing to reduced platelet adhesion on the endothelial surface under laminar flow. Conversely, VWF protein and mRNA expression were not altered by riociguat treatment. Finally, we observed that riociguat decreased P-selectin expression on the cell surface, which was associated with decreased ULVWF stabilization and platelet adhesion.

The effects of riociguat on CTEPH-PAECs were evaluated at the concentration of 0.1–1.0 µM, which was similar to the physiological plasma concentration of 150–500 nM when CTEPH patients receive up to 2.5 mg riociguat per dose. It has been reported that more than a 10-fold higher concentration is necessary to increase cGMP concentration and reduce ADP-induced platelet aggregation. As such, clinical observations of increased bleeding in riociguat-treated patients are unlikely explained by reduced ADP-induced platelet aggregation. This suggests an additional role for disturbed platelet–endothelial interaction as a cause of bleeding. The primary response to vascular trauma or injury is a repair mechanism that includes the release of VWF. Under shear conditions, VWF is unfolded and serves as a ligand for platelets to adhere to, thereby participating in wound healing. We have shown that treatment of endothelial cells with riociguat results in reduced VWF string formation and platelet adhesion. VWF expression and secretion into the circulation can be regulated via various transcriptional and (post)translational mechanisms. Although recent data from our group has shown that VWF expression in PAECs from CTEPH patients is regulated on the transcriptional level under inflammatory conditions, and that previous studies showed that mRNA levels of VWF were decreased in endothelial progenitor cells from patients receiving riociguat, we were not able to observe an anti-inflammatory effect that reduced VWF mRNA expression in PAEC after in vitro riociguat stimulation. From these results, we considered that the suppressed VWF strings formation was unrelated to VWF expression.

P-selectin is an adhesion molecule localized in WPBs in ECs and α-granules in platelets, and plays an important role in interactions between endothelial cells, platelets (aggregation), and leukocytes (cell rolling and adhesion). In WPBs, the luminal domain of P-selectin binds to the D’-D3 domains of VWF, and they are co-stored in WPBs. In response to stimulation including histamine, VWF, and P-selectin are secreted by exocytosis of WPBs on the surface of ECs, and VWF is unfolded and tethered to the endothelial surface by P-selectin, which is associated with the stabilization of secreted VWF strings against the shear stress. Our data showed that specifically P-selectin surface expression on PAECs was suppressed by riociguat treatment, which is supported by a previous study showing that an sGC stimulator (BAY 22-2727) similar to riociguat downregulates histamine-induced P-selectin expression in human umbilical vein endothelial cells. The detailed mechanism is unclear from the results of the previous and the current study. It has been reported that the endothelial secretory response is not binary and can be influenced by multiple factors, P-selectin and VWF are therefore not always co-released, which may explain our finding that P-selectin surface expression was reduced in response to riociguat, while VWF remained similar.

This study has some limitations. First, appropriate CTEPH animal models reflecting the pathogenesis of CTEPH are currently not available, which prohibits the study of the effect of riociguat on in vivo bleeding.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICS STATEMENT
This study was approved by the institutional Medical Ethical Review Board of the Amsterdam UMC, location VU University Medical Center, the Netherlands (METC Vumc, NL69167.029.19), and informed consent was obtained in accordance with the Declaration of Helsinki.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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