G-protein and Rho GTPase signaling in endothelial barrier regulation

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RhoA/B/C activation and endothelial barrier regulation under flow

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ABSTRACT

Endothelial cells (ECs) form the inner layer of blood vessels and are continuously exposed to shear forces, generated by the blood flow. These shear forces regulate EC alignment into the direction of the flow, which is required for optimal vascular integrity. While EC alignment requires dynamic Rho GTPase-mediated actin cytoskeleton remodeling, the underlying mechanisms are only partly explored. In this study we focused on the Rho subfamily of Rho GTPases (RhoA, RhoB and RhoC), and their involvement in flow-mediated signaling. By using recently-developed FRET sensors, we demonstrated that RhoA/B/C are all activated upon the induction of flow, with specific spatiotemporal characteristics. In addition, combining flow with physiologically-relevant stimuli, demonstrated sustained effects on Rho activation and endothelial barrier regulation, as compared to measurements under static conditions. Together, these data emphasize that Rho GTPases are critical determinants in endothelial signaling, and that physiological flow induces prolonged responses which affect both Rho GTPase activation, as well as the endothelial barrier.

INTRODUCTION

The endothelium consists of a single cell layer of ECs, which line the inner surface of all blood and lymphatic vessels. ECs form a semi-permeable barrier between the blood and the interstitium, facilitating the transport of oxygen, nutrients and macromolecules throughout the human body. Due to this critical transport function, ECs are constantly subjected to shear stress, which are frictional forces generated by the blood flow. Shear stress is required for the alignment of ECs into the direction of the blood flow, thereby maintaining vascular integrity. For example, in a range between 10 to 70 dynes/cm², arterial shear forces are known to be atheroprotective. Conversely, aberrant shear stress levels are related to vascular pathologies, including atherosclerosis that mainly occurs at sites of disturbed blood flow (bifurcations and curvatures).

The process of EC responses to shear forces has been described as mechanosensing and is mediated by several EC surface molecules, such as vascular endothelial cadherin (VE-cadherin), platelet endothelial cell adhesion molecule-1 (PECAM-1) and the vascular endothelial growth factor receptor-2 (VEGFR-2). Upon shear stress sensing, these molecules transduce mechanical signals into intracellular biological responses, mainly acting on the actin cytoskeleton. In turn, active cytoskeleton remodeling is required for EC alignment to the blood flow. Critical determinants in actin cytoskeleton remodeling comprises the group of Rho GTPases, which are molecular switches that cycle between an active GTP-bound and inactive GDP-bound state. Previous studies have demonstrated that both Rac1 and its functional counterpart RhoA, are involved in EC alignment to the blood flow. In addition, the use of a Förster resonance energy transfer (FRET) sensor for Rac1 has provided specific spatiotemporal
insights into Rac1 activation in flow-induced EC alignment\textsuperscript{15}. Despite the availability of a high-contrast RhoA FRET sensor\textsuperscript{16,17}, flow-induced RhoA activation has not been visualized in high detail.

In the context of Rho GTPase signaling under flow, this study focuses on the highly homologous Rho subfamily, consisting of RhoA, RhoB and RhoC. We examine RhoA/B/C expression levels as well as their activation patterns in ECs under flow. Furthermore, we combine Rho GTPase activation under flow with physiologically-relevant stimuli and their effect on vascular integrity.

RESULTS AND DISCUSSION

Physiological flow enhances RhoB expression and initiates RhoA/B/C activation in ECs

Despite their high homology in amino acid sequence (>88%), RhoA/B/C can perform unique and distinct intracellular functions (reviewed in\textsuperscript{18}). However, previous studies on flow-mediated Rho signaling have only focused on RhoA, showing that RhoA is activated upon flow-induction and is required for EC alignment\textsuperscript{14,19,20}. To further explore flow-mediated Rho signaling, ECs were grown to confluency in specialized flow chambers. In turn, a closed and pulsatile circulatory system was used to mimic the blood flow (as described in\textsuperscript{15,21}), applying arterial shear forces of 10 dynes/cm\textsuperscript{2}. Of note, within 12 hours, these shear forces are known to induce EC alignment into the direction of the blood flow\textsuperscript{15}. We compared RhoA/B/C levels in static and flow-exposed ECs and observed comparable RhoA/C levels within these two conditions (Figure 1A). While RhoB levels in static ECs are relatively low, RhoB upregulation has been documented under pro-inflammatory- and stress conditions, as well as upon growth factor stimulation\textsuperscript{22}. Here, we demonstrated that RhoB levels are also significantly upregulated in flow-exposed ECs (Figure 1A). Together, these observations imply that next to RhoA/C, also RhoB might be of great interest for shear-mediated endothelial signaling.

As indicated in the previous paragraph, all three Rho proteins are expressed in flow-exposed ECs. However, the understanding of flow-mediated Rho activation is either small or lacking. While Shiu et al. have revealed RhoA activation upon short-term flow of 30 minutes\textsuperscript{19}, this study lacked spatiotemporal data. Here we focused on a live EC approach to study RhoA/B/C activation under long-term flow conditions and EC alignment. Unfortunately, the poor quality of the differential interference contrast (DIC) images, did not allow extensive analysis of flow-induced alignment. Therefore, we limit us to RhoA/B/C activation profiles by the use of recently-developed RhoA/B/C FRET sensors (described in\textsuperscript{16,17}).

ECs were transiently transfected with one of the RhoA/B/C FRET sensors, grown to confluency in flow chambers and exposed to flow. This revealed robust activation of all three Rho proteins upon flow induction, as marked by increases in YFP/CFP ratios (Figure 1B, C). Moreover, this activation was sustained for at least 5 hrs. After these 5 hours, RhoC activation came back to baseline, while multiphasic activation patterns were observed for RhoA and RhoB.
Figure 1. ECs exposed to flow show enhanced RhoB levels and RhoA/B/C activation. A) RhoB, but not RhoA or RhoC, protein levels, detected by western blotting, were upregulated in ECs that were exposed to 12 hours of flow, 10 dynes/cm².

B) Ratiometric and DIC images of ECs that were transiently transfected with either the RhoA, RhoB or RhoC FRET sensor. Cells were exposed to flow (10 dynes/cm²) at t = 0:25 hr. Arrows on the right show the direction of the flow. Warm colors indicate high activation and correspond to the emission ratios (ER) of LUTs (look-up tables) on the right. Scale bar = 20 μm.

C) Normalized YFP/CFP traces corresponding to ratiometric images, described in B). A zoom of the first 2 hours after flow stimulation is depicted in the lower graph.
In line with our previous study, distinct spatial activation patterns were observed for RhoA/C and RhoB. While initial RhoA/C activation (at t=0:30, 5 min after flow induction) primarily localized at the cell periphery, RhoB activation was mainly initiated at perinuclear regions. In turn, sustained RhoA/B activation was concentrated at the upstream part of the cell (between t=1:00 and t=15:00). These findings are in marked contrast to the downstream-localized Rac1 activation in flow-induced EC alignment\(^4\), proposing that Rac1 and RhoA/B perform counteracting functions in flow-mediated endothelial activation and alignment. Although RhoC activation was diminished after approximately 5 hours, at t=12:00 and t=15:00 hours respectively, a small localized increase in RhoC activation was observed at the downstream side of the cell (note that the flow direction in RhoC images is opposite from the RhoA and RhoB images). Interestingly, this observation opposes the long-term spatial RhoA/B activation profiles localized at the upstream site of the cell.

Following from these findings, we can speculate about biological mechanisms that can explain the differential Rho activation patterns during flow induction and flow-induced alignment. Different Rho GTPase regulators (Rho guanine exchange factors, GEFs) are likely involved in the spatiotemporal regulation of RhoA/B/C activation. However, considering the fact that these data were acquired from single experiments in primary human cells, we first need to reproduce these findings. Conclusively, this data supports the relevance of flow-mediated Rho signaling in the endothelium, demonstrating both RhoA/B/C expression, as well as activation in ECs under flow. To what extent distinct spatiotemporal Rho activation profiles regulate biological signaling mechanisms, remains to be elucidated.

**ECs under flow show prolonged effects on barrier function and Rho GTPase activation**

In addition to the regulation of flow-mediated responses, our circulation contains numerous stimulants that act on the endothelium. One of the major stimulants in blood plasma comprises sphingosine-1-phosphate (S1P), a bioactive sphingolipid that activates three different G-protein-coupled receptors (GPCRs): S1PR1, S1PR2 and S1PR3. Although S1P promotes the endothelial barrier function via S1PR1-G\(^i\)-Rac1/Cdc42 activation, it simultaneously activates the barrier-disrupting S1PR2-G\(^{12/13}\)-Rho signaling axis\(^23\). Since these mechanisms have only been studied in static ECs, we specifically focused on S1P-mediated Rho signaling under flow.

EC monolayers with either RhoA, RhoB, or RhoC FRET sensor-expressing cells were exposed to long-term flow (for at least 12 hours) to induce EC alignment or to short-term flow (for 1 hour). Long-term flow induces EC alignment and mimics laminar blood flow in existing vessels, while short-term flow, resembles the formation of new vessels and mimics to some extent turbulent flow. In both conditions, ECs were stimulated by addition of S1P to the circulatory system, which induced sustained RhoA/B/C activation that returned to baseline levels after at least 60 min (Figure 2A-C). Remarkably, previous findings in static ECs have demonstrated that S1P-mediated RhoA/B/C activation lasts for not more than ± 5 min\(^23\), indicating that S1P-mediated RhoA/B/C signaling is prolonged in ECs exposed to flow.

Effects of S1P on endothelial barrier function can be measured by electrical cell sensing
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impedance sensing (ECIS). In static ECIS experiments, S1P stimulation coincides with a 20% increase in transendothelial resistance, that returns to baseline levels in approximately 3 hours\textsuperscript{23}. In order to investigate whether prolonged Rho signaling under flow, induced by S1P, correlates with changes in endothelial barrier function, we performed ECIS experiments under flow. EC monolayers were exposed to flow to induce EC alignment and stimulated with physiological concentrations of S1P. Compared to static ECIS experiments\textsuperscript{23}, flow-exposed ECs showed initially comparable responses to S1P stimulation, as measured by the increase in transendothelial resistance of ± 20% (Figure 2D). However, the response under flow was again sustained and barrier enhancement initiated by S1P did not return to basal levels within 7 hours. Furthermore, this prolonged effect on the endothelial barrier was not limited to S1P. This became clear after stimulating ECs which were subjected to long term flow with the protease Thrombin, which disrupts the endothelial barrier via Rho-ROCK signaling\textsuperscript{24}. Thrombin induced a robust decrease in transendothelial resistance that did not return to baseline values for approximately 10 hours (Figure 2E), while static ECIS measurement show EC-recovery of Thrombin in ± 3 hours\textsuperscript{17}.

Collectively, our data presents remarkable flow-exposed Rho activation patterns, induced by S1P. As compared to static S1P-stimulated ECs, we monitored sustained RhoA/B/C activation both in the context of blood flow in existing vessels, as well as during the formation of new blood vessels. This suggest that the blood flow critically determines temporal aspects of RhoA/B/C signaling in ECs. Although Rho signaling has been linked to cell contraction and concurrent endothelial barrier decreases via the S1PR2-G\textsubscript{12/13}-Rho signaling axis, prolonged S1P-mediated Rho activation did not result in S1P-mediated barrier decreases under the ECIS, but in fact correlated with sustained barrier stabilization. Since the S1PR2-G\textsubscript{12/13}-Rho signaling axis is counteracted by the barrier-promoting S1PR1-G\textsubscript{i}-Rac1/Cdc42 pathways\textsuperscript{23}, this suggests that also Rac1/Cdc42 signaling may be prolonged. The mechanisms by which these prolonged responses are governed are unknown. A plausible explanation might be that the continuous presence of S1P in our flow experiments affect the temporal regulation of Rho GTPase activation. Alternatively, certain key proteins might be upregulated in ECs under flow. For example, previous studies have demonstrated flow-induced S1PR1 upregulation\textsuperscript{25}. This, however, does not explain prolonged effects on Rho signaling, mediated by the S1PR2. Sustained endothelial barrier regulation is also observed after stimulation with the barrier-disrupting protease Thrombin, suggesting that this phenomenon is a general principle in EC signaling under flow, which could also relate to, for example, altered membrane dynamics and or receptor desensitization.

Overall, EC signaling under flow closely resembles the physiological situation but are also technically challenging. Over the years, static experiments in ECs have uncovered various critical mechanisms. However, we need to be aware of the fact that we are still working with endothelial model systems, and that the in vitro data from static assays are relevant to a limited extent and should be complemented by additional analysis under physiological flow conditions.
Figure 2. Prolonged Rho GTPase activation and barrier regulation in ECs under flow. A, B, C) Normalized YFP/CFP traces of ECs, transiently transfected with the RhoA, RhoB or RhoC FRET sensor, that were pre-exposed to flow (10 dynes/cm²) and subsequently stimulated with S1P (500 nM). Left graphs represent ECs that were exposed to long-term flow to induce EC alignment, right graphs represent ECs that were exposed to short-term flow. D, E) ECs were grown to a semi-confluent monolayer, exposed to flow to induce EC alignment and stimulated with D) S1P (500 nM) or E) Thrombin 1U/ml. Endothelial resistance (4000 Hz) was measured by the ECIS and normalized at t = 0. Note that for all subfigures (A-E) the onset of flow stimulation is not incorporated in the graphs.
FUTURE DIRECTIONS

Although this study is preliminary, our data proposes a critical function for RhoA/B/C signaling towards endothelial barrier regulation under flow. Since we demonstrated that Rho GTPase levels and activation in flow-exposed ECs can differ from those in static ECs, it is relevant to study this phenomenon in more detail, and for additional Rho GTPases. Besides protein analysis on Western blot, we can also include mRNA expression levels by performing qPCR analysis in flow-exposed ECs, as described in\textsuperscript{17}. Furthermore, we should not limit ourselves to Rho GTPase levels, since also S1PR levels are of great importance. Especially, since aberrant S1PR levels have been linked to vascular pathologies, e.g. atherosclerosis\textsuperscript{26,27}.

Our FRET sensor measurements under flow showed specific activation patterns for RhoA, RhoB and RhoC. We already mentioned that additional measurements are required to study these spatiotemporal responses in more detail. Interesting regulators of Rho signaling under flow comprise G-proteins, but also GEFs are of great interest. Specifically, differential localized GEFs and concurrent Rho activation, might explain perinuclear RhoB and peripheral RhoA/C activation differences. Within this context, we should not only focus on protein activity upon flow, but also assess which Rho GTPase(s) (regulators) are required for flow-induced EC alignment.

Finally, sustained signaling under flow might be a critical determinant in Rho GTPase activation and endothelial barrier regulation \textit{in vivo}. For example, pharmaceutical drugs that act on endothelial signaling pathways require strong temporal regulation, proposing flow-induced responses as highly relevant for drug discovery (e.g. in atherosclerosis). However, sustained flow-induced activation and barrier regulation should first be studied in more detail on a molecular level, to explore important players and regulatory mechanisms.

MATERIAL AND METHODS

DNA constructs
The RhoA and RhoB/C FRET sensors were described previously\textsuperscript{16,17}.

\textit{Human umbilical vein endothelial cell (HUVEC) cell culture and transfection}
Primary HUVECs, acquired from Lonza (Verviers, Belgium), were seeded on culture flasks that were pre-coated with fibronectin (FN). HUVECs were cultured in EGM-2 medium, supplemented with singlequots (Lonza), and transfected at passage #4 or #5. Plasmid DNA (2 \textmu g) was transfected by using a Neon transfection system (MPK5000, Invitrogen) and a corresponding Neon transfection kit (Invitrogen). This system generated a single pulse of 1300 Volt, for 30 ms and after electroporation, HUVECs were seeded on FN-coated culture plates.

\textit{Pulsatile flow exposure}
At least 18 hours post-transfection, ECs were transferred to FN-coated IBIDI slides (IBIDI,
Planegg, Germany) and grown to semi-confluency. In turn, after 4 to 8 hours, IBIDI slides were connected to a closed pulsatile system that mimics the blood flow, driven by a peristaltic pump (Technical University of Denmark, Kongens Lyngby, Denmark). ECs were exposed to flow rates of 10 dynes/cm² and continuously kept at 37 °C and 5% CO₂.

**Antibodies for Western blot**
Monoclonal antibodies (mAb) Rabbit anti-RhoA and Rabbit–anti-RhoC were obtained from Cell Signaling. Polyclonal antibody (pAb) Rabbit anti-RhoB was purchased from Santa-Cruz Biotechnology and mAb Mouse anti-actin was from Sigma. Secondary, HRP-labeled Swine anti-Rabbit and Goat anti-Mouse were purchased from Dako.

**Reagents**
S1P was obtained from Avanti Polar Lipids and Thrombin (HCT-0020) was from Haematologin Technologies. Both compounds were prepared according to manufacturer’s instructions.

**Live HUVEC FRET measurements**
Transfected HUVECs in IBIDI slides were prepared and stimulated as described. FRET images were acquired on a Zeiss Observer Z1 microscope. This widefield setup was equipped with a 40x oil immersion objective (NA 1.3), a HXP 120 Volt excitation light source and corresponding Zeiss/Zen 2011 software. CFP was excited at 436 (slit width, 20 nm) via a 455 dichroic longpass (DCLP (Chroma, Bellows Falls, Vermont, USA)) and emission light was directed towards a 510 dichroic shortpass (DSCP (Chroma, Bellows Falls, Vermont, USA)). In turn, a dual camera system (Hamamatsu ORCA-R2 digital CCD) allows simultaneous detection of CFP and YFP emission light. Specifically, CFP emission (455-510) was captured on the first camera via an ET 480/40 nm emission filter (Chroma, Bellows Falls, Vermont, USA), and YFP emission (>510) was captured on a second camera via an ET 540/40 emission filter (Ludl Electronics Products, NY, USA). FRET acquisitions were analyzed using a custom-made ImageJ/Fiji macro script (containing more advanced analysis options than in our studies\(^\text{17,23}\). Most notably, the script easily allows precise and cell-specific analyses of motile cells, also within dense cell populations (Ponsioen et al., unpublished) Further tools incorporated in the macro script: background subtraction (if necessary with adaptable/moving ROI), cropping, thresholding, cell-tracking, ROI-drawing.

**Electrical Cell-substrate Impedance System (ECIS)**
ECIS (Applied Biophysics, New York, USA) flow electrode arrays (1F8x10E-PC) were pre-incubated with 10mM L-Cysteine (Sigma) for 5 min at 37°C and coated with FN (10 μg/ml, 0.9% NaCl (Sigma)) for ≥1 hour at 37°C. After coating HUVECs were seeded (100.000 cells per slide) and grown to semi-confluency. In turn, cells were exposed to flow as previously described for the IBIDI slide setup. The electrical resistance was recorded at a frequency of 4,000 Hz at 37°C, 5% CO₂.
AUTHOR CONTRIBUTIONS

N.R.R. designed and performed experiments and wrote the manuscript; J.K. performed experiments; B.P. developed the FRET analysis macro; T.W.J.G. provided supervision; J.P.v.B. designed experiments; P.L.H. designed experiments and wrote the manuscript.

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