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Rivaroxaban as a Protector of Oxidative Stress-Induced Vascular Endothelial Glycocalyx Damage via the IQGAP1/PAR1-2/PI3K/Akt Pathway

Lisa Kitasato[a] Minako Yamaoka-Tojo[b] Toshiyuki Iwaya[a, c] Yusuke Murayama[a, c] Yuki Ikeda[a] Takehiro Hashikata[a] Jun Oikawa[d] Machika Suzuki[a] Nonoka Misawa[e] Rei Kawashima[e] Fumihiro Ogawa[f] Junya Ako[a]

[a]Department of Cardiovascular Medicine, Kitasato University School of Medicine, Sagamihara, Japan; [b]Department of Rehabilitation, Kitasato University School of Allied Health Sciences, Sagamihara, Japan; [c]Kitasato University Graduate School of Medical Sciences, Sagamihara, Japan; [d]Department of Kitasato Clinical Research Center, Kitasato University School of Medicine, Sagamihara, Japan; [e]Department of Regulation Biochemistry, Kitasato University School of Allied Health Sciences, Sagamihara, Japan; [f]Department of Emergency Medicine, School of Medicine, Yokohama City University, Yokohama, Japan

Keywords
Vascular endothelial glycocalyx · Endothelial cells · IQGAP1 · Rivaroxaban · Oxidative stress · PI3K/Akt pathway

Abstract
Introduction: The vascular endothelial glycocalyx, crucial for blood vessel integrity and homeostasis, is vulnerable to oxidative stress, leading to endothelial dysfunction, which strongly correlates with cardiovascular disease (CVD). This study investigates the protective effects of rivaroxaban, a factor X inhibitor, on the glycocalyx under oxidative stress condition. ***Methods:*** We examined the impact of rivaroxaban on human umbilical vein endothelial cells exposed to acute and chronic H_2O_2-induced oxidative stress. ***Results:*** Rivaroxaban dose-dependently suppressed syndecan-1, a key component of the glycocalyx, shedding from cell surface, and enhanced protease-activated receptor (PAR)1-PAR2/phosphatidylinositol-3-kinase (PI3K)-dependent cell viability after acute induction of H_2O_2. This protective effect was linked to the translocation of IQGAP1, a scaffold protein that modulates the actin cytoskeleton, to the perinucleus from the cell membrane. Under chronic H_2O_2 treatments, rivaroxaban improves cell viability accompanied by an increase in hyaluronidase activities, aiding the turnover and remodeling of hyaluronic acid within the glycocalyx. ***Conclusion:*** We identify that rivaroxaban protects against oxidative stress-induced endothelial glycocalyx damage and cell viability through IQGAP1/PAR1-2/PI3K/Akt pathway, offering a potential to be a therapeutic target for CVD prevention.

Introduction

Endothelial cells (ECs) are surrounded by a negatively charged gel-like layer, the glycocalyx, which serves as a physical barrier between blood and vessel walls as well as a buffer region between cells and the extracellular matrix

karger@karger.com
www.karger.com/jvr

Correspondence to:
Lisa Kitasato, lisa@med.kitasato-u.ac.jp

[1]. The vascular endothelial (VE) glycocalyx consists of glycosaminoglycan side chains (e.g., heparan sulfate, chondroitin sulfate, etc.), long-chain hyaluronic acid (HA) and proteoglycans (e.g., syndecan-1 and CD44) [2, 3]. Syndecan-1 is primarily involved in the structural stabilization of the glycocalyx, which serves as a barrier against vascular leakage and a regulator of cell adhesion and permeability [4]. Syndecan-1 interacts with coagulation factors [5, 6] like tissue factor and tissue factor pathway inhibitor (TFPI) [7], modulating coagulation and influencing the initiation of the extrinsic coagulation cascade. These interactions are crucial for preventing excessive coagulation and thrombosis on the endothelial surface. Additionally, syndecan-1's interaction with protease-activated receptors (PARs) [8, 9], particularly PAR1 and PAR2, which are activated by thrombin and other proteases during coagulation and inflammatory responses, plays a significant role in modulating cellular responses such as barrier function, inflammation, and wound healing [10].

The glycocalyx is involved in vascular integrity and homeostasis by regulating many physiological processes, such as coagulation, inflammation, vasocontraction and relaxation, vascular permeability, and angiogenesis [11, 12]. Damage to the endothelial glycocalyx decreases vascular barrier function and leads to protein extravasation and tissue edema, loss of nutritional blood flow, and an increase in platelet leucocyte adhesion [13–16]. VE glycocalyx shedding and degradation are known to be caused by a variety of cellular stressors [17]. Specifically, inflammatory mediators [18], hypoxia or excessive reactive oxygen species [19], ischemia/reperfusion injury [20], atrial natriuretic peptide, and endotoxin [21] are the cause of VE glycocalyx damage. Perturbed glycocalyx aggravates endothelial dysfunction, causing reduced nitric oxide bioavailability [22], inflammatory cytokine release [23], platelet adherence [24], coagulation [25], and leukocyte adhesion [26]-pathologies that are deeply associated with cardiovascular disease (CVD). VE glycocalyx degradation exacerbates inflammatory mechanisms that promote tissue degradation, including reactive activation of matrix metalloproteinases (MMPs), heparanases, and hyaluronidases. Regeneration of glycocalyx has emerged as a critical target for understanding and potentially treating CVD [27]. In addition to chronic CVD such as heart failure [28], atherosclerosis, diabetes [29] or Kawasaki disease [30], loss of the glycocalyx is implicated in acute shock states, such as acute coronary syndrome [31], hemorrhage and septic shock [32, 33]. Noting the potential for pharmacotherapy of both acute and chronic pathology might be useful.

On the other hand, we have clarified that rivaroxaban, an activated blood coagulation factor X (FXa) inhibitor, exerts its effects through multiple pathways [34–36]. Primarily, as an anticoagulant, rivaroxaban directly inhibits FXa through FXa-PARs signaling, which is crucial in the coagulation cascade, thereby preventing the conversion of prothrombin to thrombin. This inhibition reduces thrombin generation and consequently diminishes thrombin-mediated activation of PARs [37]. We have previously demonstrated that rivaroxaban has not only an anticoagulant effect but also an antiapoptotic and anti-inflammatory effect by inhibiting nuclear-factor-kappa B signaling [34, 35]. A recent study reported that human ECs produce their own coagulation factors that can activate cell surface FX without the addition of exogenous proteins or phospholipids [38] suggesting that the modulation of the FX activity is an important biological process in ECs. As a consequence, we tested for the first time the hypothesis that rivaroxaban protects against endothelial glycocalyx damage.

To explain this protective effect, we studied the link between rivaroxaban activity and phosphatidylinositol-3-kinase (PI3K)/serine-threonine protein kinase (Akt) pathway. Indeed, it is already known that the PI3K/Akt pathway is linked to the VE glycocalyx as it essential for EC survival by activating HA/CD44 [39]. Moreover, it is also known that HA activates the HA/CD44 system by binding CD44 and regulates various signaling pathways through IQ motif-containing GTPase activating protein 1 (IQGAP1). IQGAP1, a scaffold protein that binds to VE growth factor receptor type 2 (VEGFR-2), modulates the actin cytoskeleton, cell-cell adhesion, cellular motility, and morphogenesis. HA-induced CD44-IQGAP1 complex is capable of binding F-actin and regulates cytoskeletal function [40]. Furthermore, IQGAP1 colocalizes and forms a complex with VE-cadherin at the site of cell-cell contact and link VEGFR-2 to the VE-cadherin/βcatenin complex at the human umbilical vein endothelial cell (HUVEC) adherens junctions which may contribute to angiogenesis [41]. IQGAP1 is essential to control the modulation of cytoskeletal architecture and other important physiological function in the vasculature [40].

According to previous study, glycocalyx layer around 1,5-2 μm is observed in cultured HUVECs even in static condition [42]. Hydrogen peroxide is a well-established inducer of oxidative stress in various cell types, including ECs. Numerous studies have demonstrated its efficacy in mimicking oxidative stress conditions that lead to VE dysfunction, including increased endothelial permeability, barrier dysfunction, and apoptosis [43, 44]. In the

present study, we analyzed the effect of rivaroxaban and the relation with PI3K/Akt/IQGAP1 pathway in HUVECs under excessive oxidative-stress conditions.

Methods

Cell Culture

HUVECs (Promo Cell C12203) were cultured at 37°C with 5% CO_2 in endothelial growth medium 2 (EGM2, Lonza CC4176). Cells between passages 3–5 were then seeded at 8,000 cells/cm^2, in collagen Type 1 (Sigma–Aldrich C8919, St. Louis)-precoated 96-well plates, 10 cm plate dishes or Lab-Tek slides (Nunc 177437PK, Waltham) until confluence. HUVECs were starved pretreated with or without rivaroxaban (1 μg/mL, approximately 2.29 μM, unless indicated otherwise, Toronto R.C R538000, Toronto) in 0.5% FBS (Sigma F7524, St. Louis) endothelial basal media (EBM2, Lonza CC3156, Basel) for 1 h and stimulated by H_2O_2 (Wako 086–07445, Osaka) 1 mM (unless indicated otherwise) in 0.5% FBS in EBM for the designated hours.

Syndecan-1 Level Measurement

To detect the level of syndecan-1 in the culture supernatant, cells were starved and treated as described in the previous section. Cell culture supernatants (150 μL/well of 96-well plate) were collected, centrifuged at 1,000 × *g* for 10 min, and was immediately use. The cell lysates were also collected using RIPA buffer (Wako 188-02453, Osaka) (150 μL/well of 12-well plate), followed by a ultrasonication technique, and stored at −80°C. The concentration of soluble syndecan-1 (sCD138) was measured by ELISA (Diaclone 950.640.192, Besançon) at the dilution of 1:1.

Quantification of FX in HUVECs Lysates

FX levels in ECs were measured as previously described [38]. Briefly, cell lysates were prepared from HUVECs grown in 10 cm dishes until confluence in EGM2. 24-h prior to lysate preparation, cells were washed 3 times with Ca^{+2}, Mg^{+2}-containing PBS and were then starved for 24 h in serum-free medium (EBM, Lonza CC3156, Basel + insulin-transferrin-selenium, Thermo Fisher 41400045, Waltham) alone, stimulated with or without H_2O_2 1 mM. To ensure that all traces of exogenous proteins are eliminated, after removing the media, cells were washed 3 times with 10 mL of cold, sterile Tris buffer (50 mM Tris, pH 7.3, 1% BSA, and 5 mM $CaCl_2$). Then, on ice, cells were lysed with a lysis buffer (pH 7.4 10 M Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) + protease inhibitor (Wako 165-26021, Osaka) 167 μL for 15 min with rocking. Cells were collected with a cell scraper, followed by a ultrasonication technique, place into a chilled tube and centrifuged at 12,000 *g* for 15 min at 4°C. The cell supernatants were collected and stored at −80°C until assay. The protein was quantified with undiluted lysates using FX ELISA kit (Abcam ab108832, Boston).

Immunofluorescence and Quantification Analysis

After treatments, cells were fixed with 1% paraformaldehyde for 10 min and permeabilized with 0.2% Triton +50 mM NH_4Cl for 10 min. Cells were then incubated overnight with IQGAP1 conjugated to Alexa Fluor 488 (1:50, Santa Cruz 3760211, Dallas), VE-cadherin conjugated to Alexa Fluor 546 (1:100, Santa Cruz 9989, Dallas) or mouse control IgG (1:20,000, Jackson Immuno Research 015-000-003, West Grove). Thereafter, the samples were incubated with secondary antibody for the control IgG and DAPI nuclear stain (Thermo Scientific 62248, Waltham), covered with mounting medium (Diagnostic BioSystems K024, Pleasanton). Multichannel images were acquired with an inverted confocal microscope using a Plan-Apochromat 63X/1.4 Oil DIC objective. The images were Z-projected, analyzed with ImageJ software (NIH, Bethesda). Data are expressed as the means of the fold change +/− SE of the means of the fluorescence intensity from the protein of interest.

Quantification of Cell Viability

The MTT assay was performed using a kit (Roche 11465007001, Basel) as previously described [45] in the presence of rivaroxaban 1 μg/mL followed by 1 mM-H_2O_2 stimulation for 20 min. Control experiments were performed in parallel. To explore the participation of key signaling modulators, cells were pretreated with pharmacological inhibitors for 1–2 h before H_2O_2 stimulation. Specifically, the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 at 20 μM (Sigma–Aldrich L9908, St. Louis), the PAR1 antagonist SCH79797 at 200 nM (Tocris Bioscience 1592, Bristol) and the PAR2 antagonist AZ3451 at 200 nM (Tocris Bioscience 6806, Bristol).

Profiled Protein Array

Confluent HUVECs cultured in 10 cm dishes (4.0×10^6 cells/dishes) were treated with or without rivaroxaban followed by stimulation with H_2O_2. Cells were lysed and total protein was extracted with lysis buffers (R&D Systems, Minneapolis). Finally, proteins were analyzed on cell lysates with the Proteome Profiler Human Protease Array

kit (R&D Systems ARY009, Minneapolis) and Proteome Profiler Human Apoptosis Array kit (R&D Systems ARY021B, Minneapolis) according to the manufacturer's instructions. Spots were quantified with IQ800 (Cytvia, Marlborough), and values were reported as the mean pixel density. For data analysis, two-way ANOVA (factor1 = treatment, factor2 = protein) was first performed, multiple comparison was only made for treated cells versus untreated cells (control), followed by a Dunnet post hoc analysis. Multiple unpaired *t* test was then performed for each protein (parametric test, post hoc test = Holm-Sidak method) using no treated cells as control each time to define a list of protein affected by the treatment. Identified significant proteins were analyzed on reatome.org, followed by network analysis using STRING database, which allowed for the clustering of the selected proteins into the main relevant protein networks (signaling pathway or protein functions) in which they are involved [46].

MMP Activity and Hyaluronidase Activity Assay

The MMP and hyaluronidase activities in cell culture media from the cells treated as described above were measured using the Sensolyte 390 generic MMP activity kit (AnaSpec 71158, Fremont) and the hyaluronidase activity ELISA kit (Echelon Biosciences K-6000, Salt Lake City) following the manufacturer's instructions. For MMP activities, the fluorescence intensity of Mca fluorescence standard and enzyme reaction was measured at Ex/Em = 300 nm/390 nm. Mca fluorescence reference standard curve was plot as RFU (relative fluorescent unit) versus concentration.

Statistical Analysis

Statistical analyses (except for Protein Array analysis which is described above) were performed using JMP 16 (SAS Institute). Data are presented as the mean +/− SE. Continuous variables were compared using the one-way ANOVA and Dunnett's post hoc analysis. *p* value of <0.05 was considered statistically significant.

Results

Soluble Syndecan-1 Release Is Dose-Dependently Inhibited by Rivaroxaban in ECs under Oxidative Stress

VE glycocalyx degradation occurs in the presence of excessive ROS [22]. Evidence suggests that HUVECs viability was at 70% in 0.2 mM and 40% at 1 mM H_2O_2 after 0, 5-2 h of exposure [47, 48]. We therefore tested 1–100 mM as range of H_2O_2 concentration. As expected, the concentration of soluble syndecan-1 (sCD138, a marker of VE glycocalyx damage) after 20 min of H_2O_2 stimulation in cultured ECs was significantly higher in a dose-dependent manner, accompanied with the decrease of cell viability than that in unstimulated cells (Fig. 1a, b).

The sCD138 levels in cell lysates were also measured to detect the shed syndecan-1, which could be taken up by cells [49] or which remains in the cell membrane. There was no change in sCD138 concentration in all conditions whether with or without H_2O_2 stimulation and with or without rivaroxaban pretreatment (mean 22.1+/− 1.05 pg/mL), suggesting that the role of shed syndecan-1 in the cells or its expression in the cell membrane may be insignificant in our study model.

To examine the drug effect of FXa inhibition, we pretreated cells with or without rivaroxaban, having confirmed ourselves that HUVECs produced their own FX and that this production was 1.9-fold higher when treated with H_2O_2 (Fig. 1c). Rivaroxaban suppressed H_2O_2-induced syndecan-1 shedding from the cell surface in a dose-dependent manner (Fig. 1d).

Involvement of the PAR1/PAR2 Pathway on the Cell Viability in the Acute Phase

Since we observed the protective effect of rivaroxaban on the inhibition of glycocalyx impairment in the acute phase under oxidative stress, we examined whether cell viability was also preserved. Analysis of the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction assay 20 min after H_2O_2 stimulation, showed an increase in cell viability in rivaroxaban-treated ECs compared to untreated cells (Fig. 2). Because FXa regulates the activation of extracellular proteases, such as thrombin, activation of the protease-activated receptors (PARs) family can be involved in the protective effect of rivaroxaban. Thus, the implication of PAR1 and PAR2 receptors was explored in H_2O_2-induced cell damage with or without the presence of rivaroxaban. As shown on Figure 2, the inhibition of PAR1 or PAR2 alone was not sufficient to recover the H_2O_2-induced cell damage but the addition of rivaroxaban was able to recover the cell viability in both conditions. Blocking both PAR1 and PAR2 was required to protect from H_2O_2 damage, while neither antagonist alone had this effect (Fig. 2). These data suggest that the inhibition of both PAR1 and PAR2 is required to rescue H_2O_2-induced cell viability.

Protein Profiling

To further decipher the mechanism underlying the protective effect of rivaroxaban, Proteome Profiler Array kits were used to quantify changes in proteins related to apoptosis and cell stress in H_2O_2-stimulated cells, with or without rivaroxaban pretreatment. The treatment with

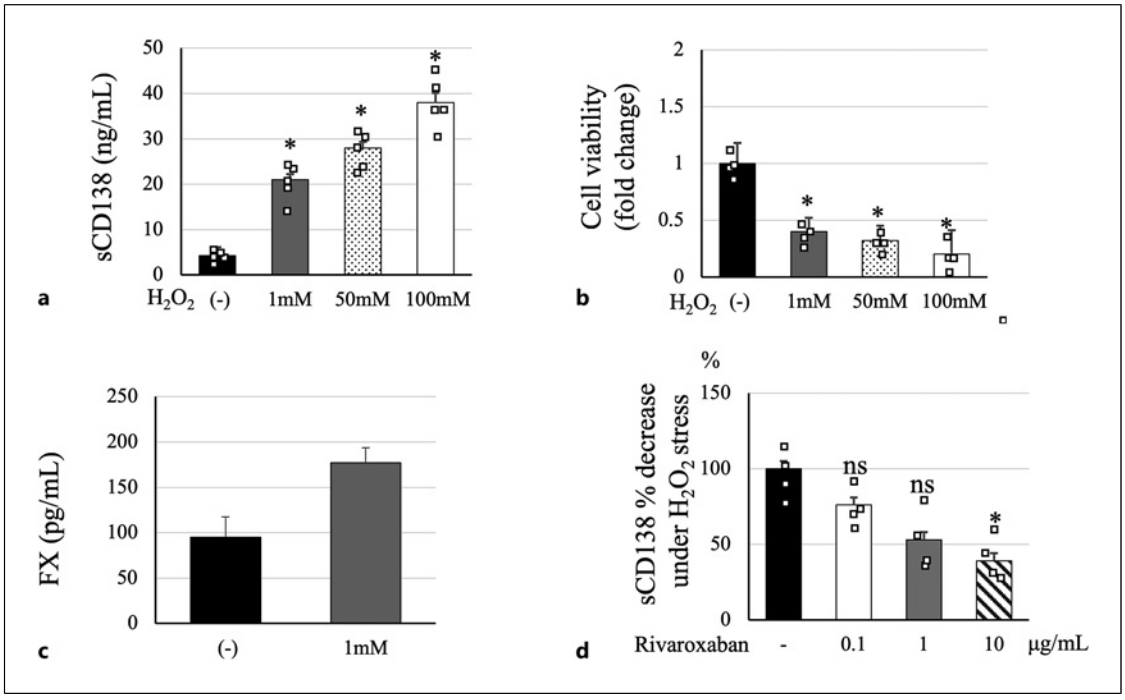

Fig. 1. sCD138 release under oxidative stress and the effect of rivaroxaban in ECs. **a** sCD138 concentration in the culture supernatant of HUVECs 20 min after 1, 50, 100 mM H_2O_2 stimulation (n = 5 for each culture). **b** ECs viability in 20 min after 1, 50, 100 mM H_2O_2 stimulation (n = 4 for each culture). **c** FX levels measured in HUVEC cell lysates with or without 20 min H_2O_2 treatment (n = 2 for each culture). **d** sCD138 concentration in the 1 mM H_2O_2-stimulated HUVEC culture supernatant with or without rivaroxaban pretreatment (n = 4 for each culture). Bar graph represents averaged data. Values are the mean +/−SE. *p < 0.05 versus control. Significance is assessed using one-way ANOVA, followed by Dunnett's post hoc analysis. ECs, endothelial cells; sCD138, soluble CD138; HUVECs, human umbilical vein endothelial cells.

H_2O_2 induces significant changes in the abundance of the tested proteins, suggesting that H_2O_2 treatment induces apoptosis. In contrast, no significant differences were observed with rivaroxaban alone or when H_2O_2 treated cells were also treated with rivaroxaban (Fig3a, b; see online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000542419 for the originally saved images). This strongly suggests that rivaroxaban protects against apoptosis. There was no significant change in multiple unpaired t test for each protein.

For cell stress-related proteins, the treatments with H_2O_2 or rivaroxaban alone did not induce significant differences in the abundance of the tested proteins (Fig. 3c). However, significant differences were observed when cells were treated with both H_2O_2 and rivaroxaban. This emphatically suggests that co-treatment with both agents may induce changes in the abundance of proteins related to stress. COX-2, p21, SOD2, Thioredoxin, and BCL2 were identified in multiple t test (Fig. 3d).

These proteins were analyzed (overrepresentation analysis) on reactome.org (including interactors, used in 2024-05-30). The Table 1 is the 10 most significant pathways found. Among them, the PI3K/AKT pathway seems to be important (2nd, 3rd, and 9th pathways in Table 1). The protein networks are shown in Figure 3f.

The Involvement of PI3K Signaling on the Rivaroxaban-Mediated Soluble Syndecan-1 Shedding and Cell Viability in Acute Phase

Based on our observations in protein profiling, we then verified if the PI3K/Akt pathway can modulate the effect of rivaroxaban. Improvements in cell viability (Fig. 4a) and sCD138 release (Fig. 4b) mediated by rivaroxaban in ECs were downregulated by the addition of a PI3K inhibitor (LY29002) indicating that PI3K/Akt seems to be involved in the protective effect of rivaroxaban.

Rivaroxaban and Glycocalyx-Associated Factors in Chronic Phase

To further clarify the chronological changes in H_2O_2-stimulated syndecan-1 release, we analyzed the sCD138 concentration in the culture supernatant and other EC

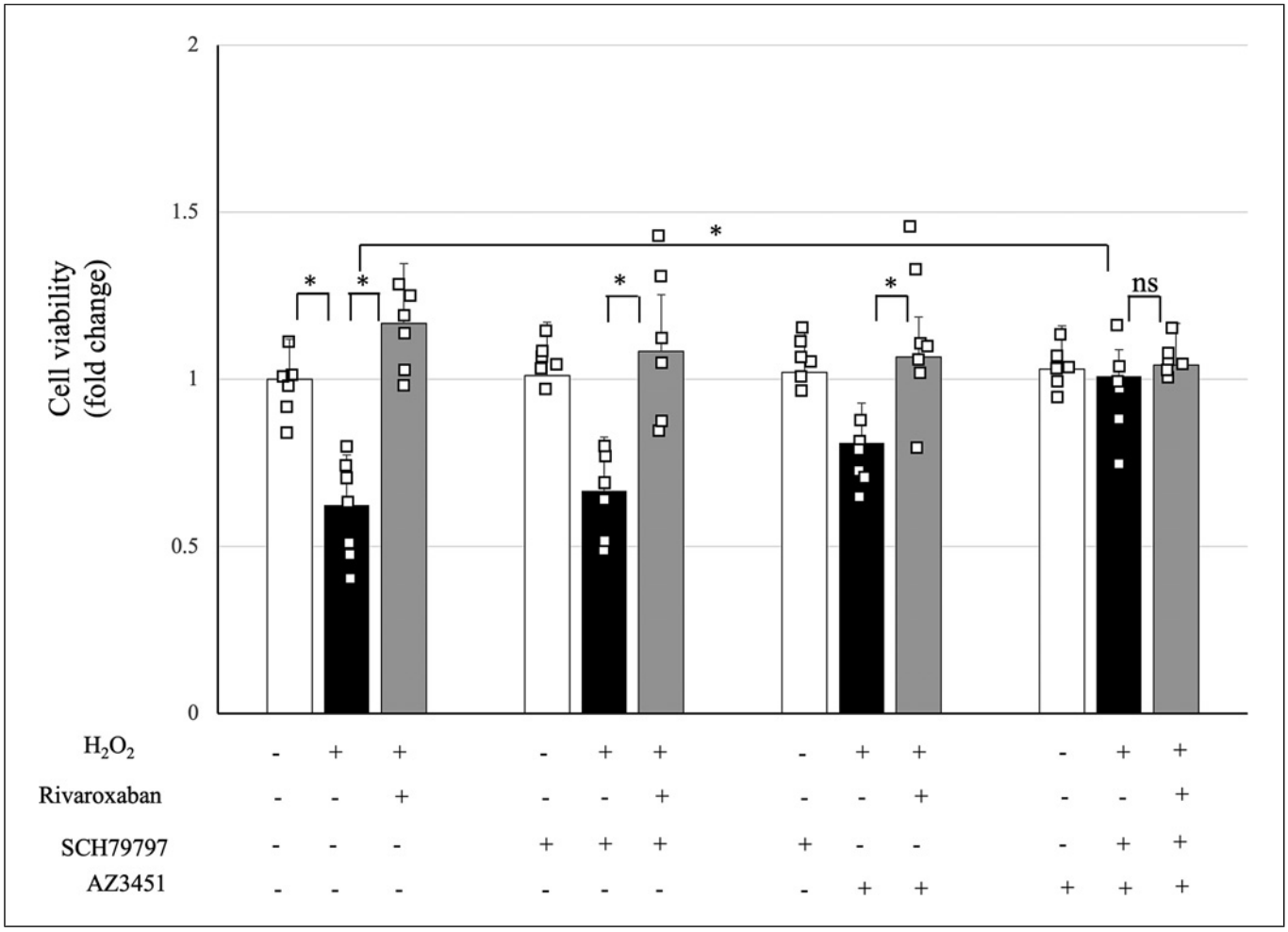

Fig. 2. Rivaroxaban improves cell viability in the acute phase via the PAR1/PAR2 receptors ECs viability in 20 min of 1 mM H_2O_2 stimulation with or without rivaroxaban. Treatment with PAR1 antagonist SCH79797, PAR2 antagonist AZ3451, or their combination as indicated ($n = 6$ for each culture). Bar graph represents averaged data. Values are the mean +/−SE. *$p < 0.05$. Significance is assessed using one-way ANOVA, followed by Dunnett's post hoc analysis. ECs, endothelial cells; PAR, protease-activated receptor.

glycocalyx-associated factors that could contribute to the chronic phase. After 12 and 24 h of 1 mM H_2O_2 stimulation, sCD138 was no longer detectable in the culture supernatant, suggesting the degradation of the shed EC glycocalyx by oxidative stress (data not shown). However, ECs pretreated with rivaroxaban accompanied an amelioration of cell viability (Fig. 5a). Rivaroxaban pretreatment also demonstrated a greater increase in hyaluronidase activity (Fig. 5b). In the vasculature, hyaluronan is mainly incorporated into the glycocalyx [2, 3]. The degradation of a small amount of hyaluronan (10–100 mg/day in the blood of adult humans) can be considered as its turnover, and this process is highly dependent on the activity of hyaluronidases, a family of enzymes that degrade HA [29]. This enhanced remodeling process could help to clear damaged components of the glycocalyx, thus maintaining its barrier function and protective properties against oxidative stress and inflammatory mediators. By facilitating the regulated turnover of hyaluronan and possibly other glycosaminoglycans within the glycocalyx, rivaroxaban could help preserve the essential permeability barrier and mechanotransduction functions of the endothelial layer. Furthermore, the removal of old or oxidatively damaged hyaluronan through increased hyaluronidase activity may prevent the accumulation of dysfunctional glycocalyx components, which could otherwise lead to increased vascular permeability and leukocyte adhesion,

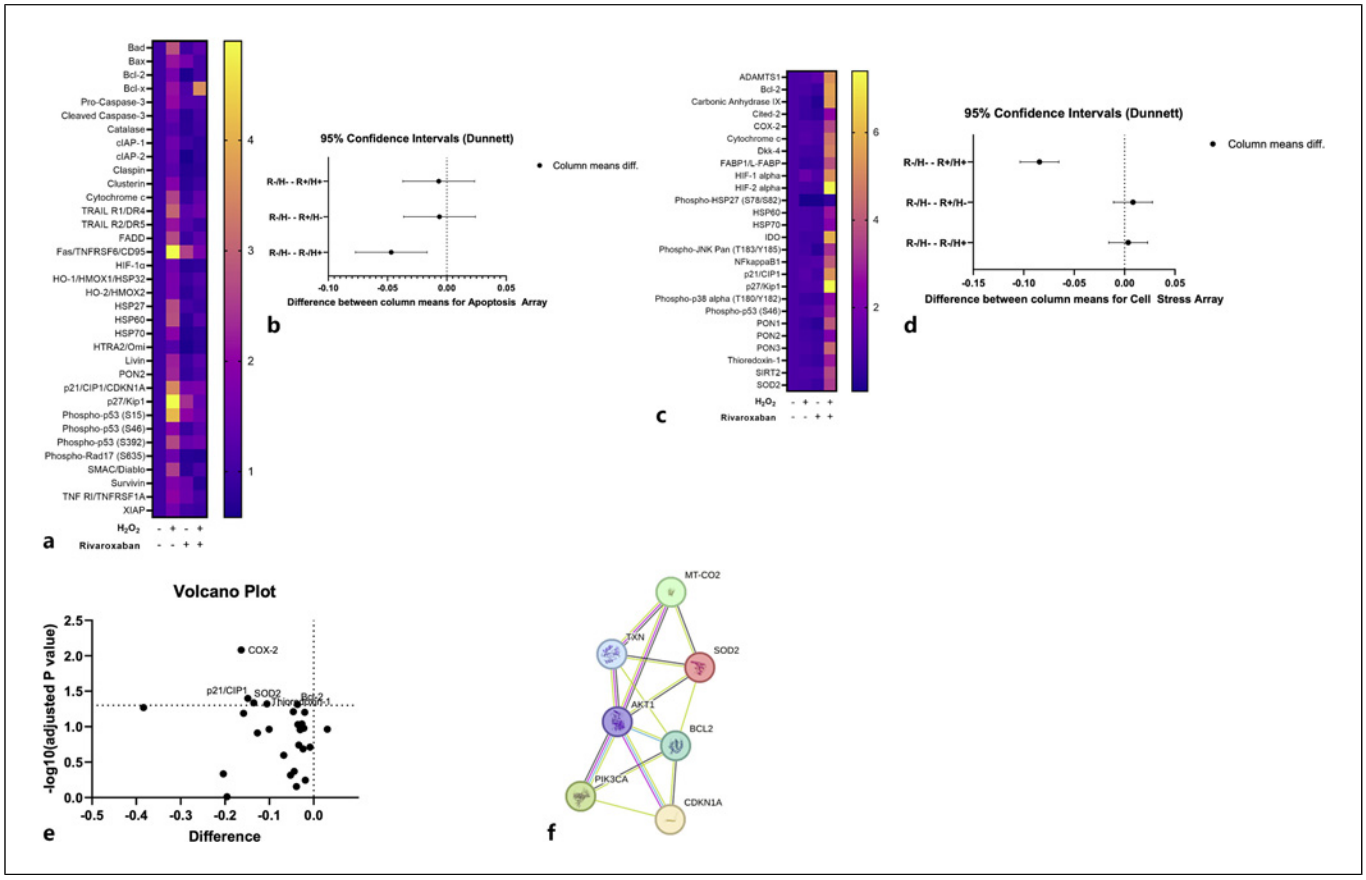

Fig. 3. Cell apoptosis and cell stress array. **a** Heatmap of cell apoptosis array. Heatmap of apoptosis-related proteins was generated from the list of indicated proteins. The effect of rivaroxaban in ECs for the list protein was analyzed 20 min after the 1 mM H_2O_2 stimulation. The relative value for each protein is depicted by color density, with yellow indicating upregulated and purple downregulated proteins. **b** 95% confidence intervals for apoptosis array. Difference between column means of heatmap was analyzed. **c** Heatmap of cell stress array. Heatmap of cell stress-related proteins was generated from the list of indicated proteins. The effect of rivaroxaban in ECs for the list protein was analyzed 20 min after the 1 mM H_2O_2 stimulation. The relative value for each protein is depicted by color density, with yellow indicating upregulated and purple downregulated proteins. **d** 95% confidence intervals for cell stress array. Difference between column means of heat map was analyzed. **e** Volcano Plot of cell stress array. Each protein was compared by multiple *t* test. The name of significative proteins is indicated. **f** The protein network for cell stress array analyzed in STRING database. In green: text mining interaction, in purple: experimentally determined interactions, in blue: from curated databases interactions. ECs, endothelial cells; RVX, rivaroxaban; SOD, Superoxide dismutase; CDKN1A, cyclin-dependent kinase inhibitor 1; PIK3CA, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform; MT-CO2, cytochrome c oxidase subunit 2; TXN, thioredoxin; AKT, alpha serine/threonine-protein kinase.

exacerbating inflammatory processes. Taken together, our results suggest that rivaroxaban could also have a protective role during the turnover of the EC.

A previous report has shown that the loss of syndecan-1 is associated with suppressed MMP activity, which is regulated by sphingosine 1-phosphate (S1P)-dependent pathways [50]. However, in our study, although H_2O_2-induced MMP activities, these activations were not significant despite the addition of rivaroxaban (Fig. 5c), suggesting that a mechanism other than the regulation of MMPs modulates the protective effect of rivaroxaban on the EC glycocalyx.

Rivaroxaban Protects against Weak Cell-Cell Adhesion Induced by Oxidative Stress through IQGAP1 Scaffolding

Our observations indicate that rivaroxaban-augmented endothelial glycocalyx protection is regulated by a process other than the activation of MMPs but is modulated by the PI3K/Akt pathway. It has been shown that the PI3K/Akt pathway is essential for EC survival by activating HA/CD44 [39]. Moreover, HA activates the HA/CD44 system by binding CD44 and regulates various signaling pathways through IQ motif-containing GTPase

Table 1. The most significant pathways in cell stress array

Pathway name	Entities				Reactions	
	found	ratio	*p* value	FDR	found	ratio
FOXO-mediated transcription	3	0.006	9.07 E-06	0.003	5	85
Constitutive signaling by AKT1 E17K in Cancer	1	0.002	2.08 E-05	0.003	15	18
PI3K/AKT signaling in cancer	1	0.010	5.16 E-05	0.004	15	21
Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models	1	0.002	8.92 E-05	0.004	6	22
Neurodegenerative diseases	1	0.002	8.92 E-05	0.004	6	22
Defective intrinsic pathway for apoptosis	1	0.002	8.98 E-05	0.004	6	24
Cellular response to chemical stress	4	0.018	9.62 E-05	0.004	9	166
Diseases of programmed cell death	1	0.007	1.18 E-04	0.005	6	37
Cellular responses to stress	5	0.067	2.14 E-04	0.007	16	486
Cellular responses to stimuli	5	0.068	2.25 E-04	0.007	16	517

The 10 most relevant pathways were sorted by *p* value in reacctome.org. FDR, false discovery rate.

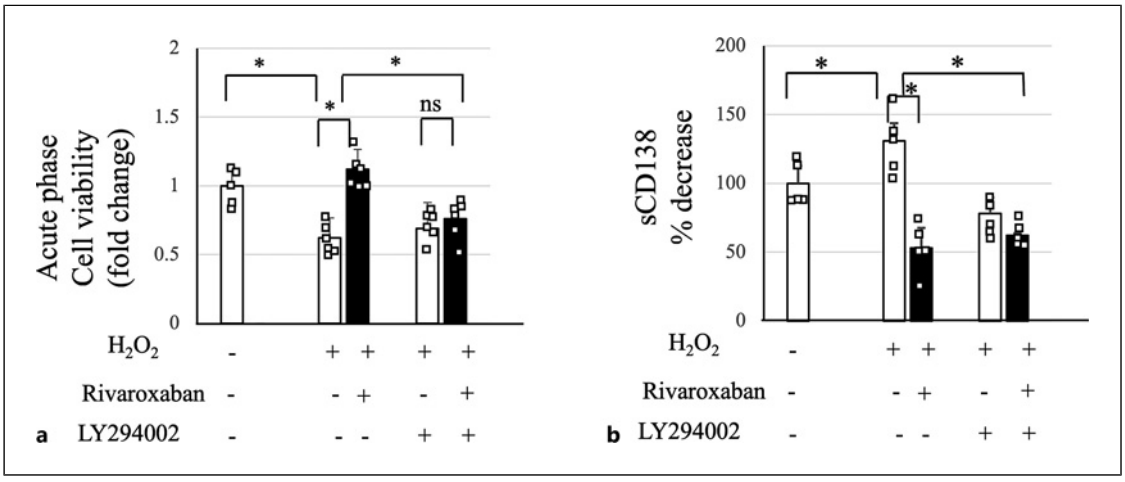

Fig. 4. Involvement PI3K pathway in the improvement of cell viability and sCD138 shedding by rivaroxaban. **a** The effect of rivaroxaban in ECs viability in 20 min of 1 mM H_2O_2 stimulation with or without PI3K inhibitor LY29002 (n = 5 for each culture). **b** The effect of rivaroxaban on sCD138 shedding from the cell surface was measured using HUVEC culture supernatant in 20 min of H_2O_2 stimulation with or without LY29002 (n = 5 for each culture). Bar graph represents averaged data. Values are the mean +/−SE. *$p < 0.05$. Significance is assessed using one-way ANOVA, followed by Dunnett's post hoc analysis. ECs, endothelial cells; PI3K, phosphatidylinositol 3-kinase.

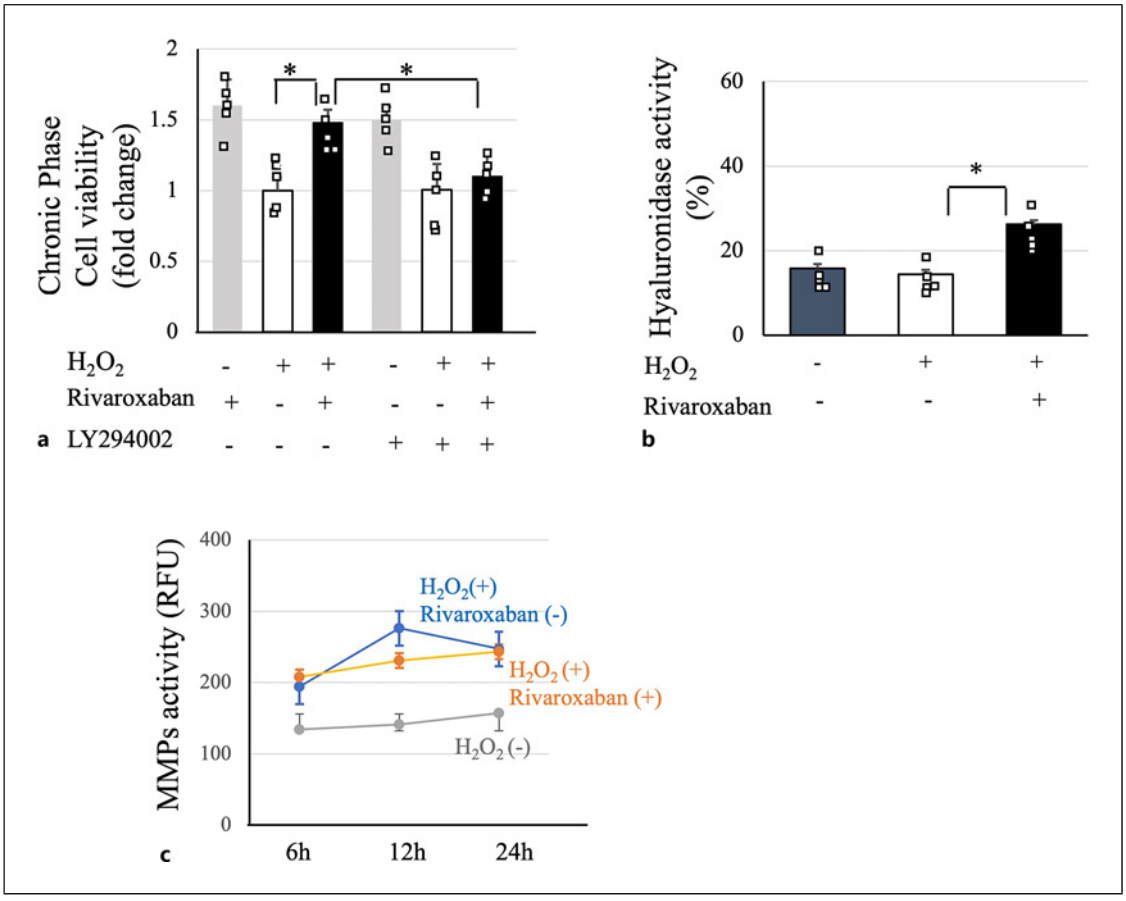

Fig. 5. Glycocalyx-associated factors after long-term H_2O_2 stimulation. **a** MTT assay after 24 h of H_2O_2 stimulation in HUVECs (n = 5 for each culture). **b** Hyaluronidase activity after 24 h of H_2O_2 stimulation in HUVECs (n = 5 for each culture). **c** MMPs activities after 6, 12, 24 h of 1 mM H_2O_2 stimulation in HUVECs (n = 5 for each culture). Bar graph represents averaged data. Values are the mean +/−SE for 3 independent experiments. *p < 0.05 versus control. Significance is assessed using one-way ANOVA, followed by Dunnett's test. ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; MTT, (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), MMPs; matrix metalloproteinases.

activating protein 1 (IQGAP1). For example, IQGAP1 interacts with CD44 and promotes cell proliferation, cell migration, angiogenesis, cell survival, and cell differentiation [51]. Thus, we then focused on this scaffold protein, IQGAP1. As a scaffold protein involved in different signaling pathways (actin cytoskeleton [51], cellular adhesion [52], cell cycle, and transcription), IQGAP1 has a unique ability to potentially couple diverse cellular functions. For instance, IQGAP1 is associated with actin dynamics through direct binding of actin and indirect regulation through Cdc42/Rac1 [53].

We examined the subcellular localization of IQGAP1 and VE-cadherin before and after 5 min of H_2O_2 stimulation with or without rivaroxaban pretreatment in a confluent monolayer of HUVECs using confocal microscopy. As shown in Figure 6a, in unstimulated ECs, IQGAP1 was mainly found at cell-cell contacts, where it colocalized with VE-cadherin. H_2O_2 stimulation reduced the staining of both VE-cadherin and IQGAP1 at cell-cell contacts, consistent with the loss of cell adhesions, whereas it increased the staining of IQGAP1 and VE-cadherin at the perinuclear area. Strikingly, rivaroxaban inhibited H_2O_2-induced IQGAP1 translocation from the cell membrane to the perinucleus and was diffusely distributed within the cytosol (Fig. 6a, b). Interestingly, in unstimulated ECs, IQGAP1 was relocalized in the nucleus when treated with rivaroxaban (Fig. 6a, c). There was no specific binding in cells incubated with control IgG (Fig. 6a). Taken altogether, our results strongly suggest, that the protective effect of rivaroxaban is mediated by the PI3K/Akt/IQGAP1 pathway under excessive ROS-induced vascular cell damage conditions.

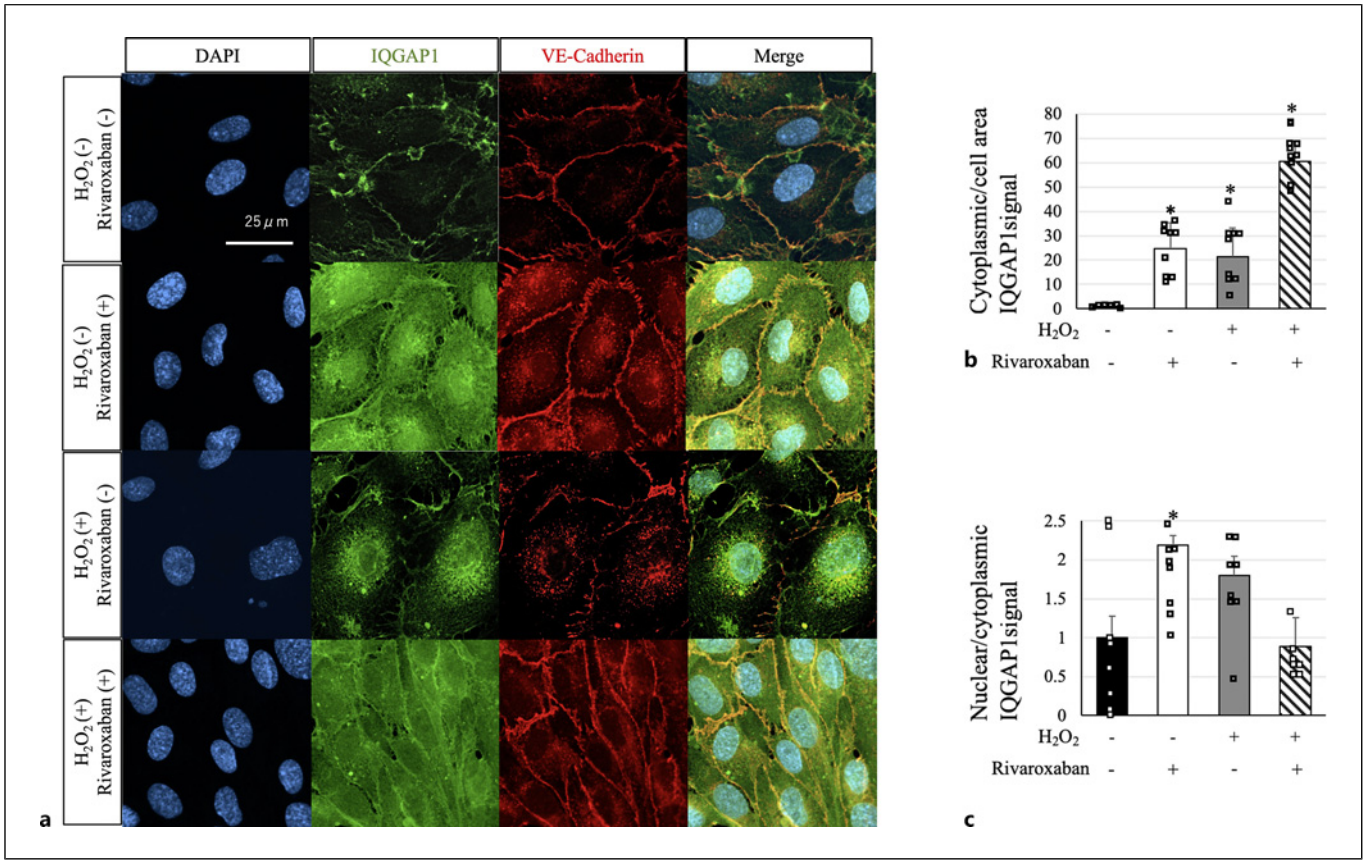

Fig. 6. Rivaroxaban inhibits H_2O_2-induced IQGAP1 translocation from the EC membrane to the perinucleus. **a** Immunodetection of IQGAP1 and VE-cadherin after 5 min of 1 mM H_2O_2 stimulation in HUVECs. Mouse control IgG were incubated with secondary antibodies: donkey-anti mouse IgG (Alexa Fluor 594) and goat-anti mouse IgG (Alexa Fluor 488). **b** Quantification of IQGAP1 cytoplasmic signal to the cell area (n = 8 cells/conditions). **c** Quantification of nuclear IQGAP1 normalized to the cytoplasmic signal (n = 8 cells/conditions). Values are the mean +/−SE for 3 independent experiments. *$p < 0.05$ versus control. Significance is assessed using one-way ANOVA, followed by Dunnett's test. IQGAP1, IQ motif-containing GTPase activating protein 1; EC, endothelial cells; VE-cadherin, vascular endothelial cadherin, DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride.

Discussion

Our observations suggest that rivaroxaban has a protective effect against oxidative stress-induced glycocalyx damage, improving cell viability and preserving the integrity of VE-cadherin interactions. To the best of our knowledge, this is the first work to propose a comprehensive mechanism for endothelial glycocalyx protection by rivaroxaban.

As damage to the endothelial glycocalyx reduces vascular barrier function and results in several physiological perturbations, preserving, and restoring the endothelial glycocalyx is a potential target to prevent CVD [27]. Here, we propose rivaroxaban, as a novel protector. Previous studies have shown that metformin, a hypoglycemic drug, or sulfoxide, a heparin sulfate-like compound, improve he endothelial glycocalyx in diabetic mice or in septic mice, respectively [54, 55]. Hydrocortisone inhibits mast-cell degranulation, tumor necrosis factor-*a* inhibitor direct antagonizes glycocalyx degradation, avoidance of high-volume loading prevents the liberation of natriuretic peptides, and infusion of human plasma albumin maintains stability of the EC surface layer and has been also reported to help reduce oxygen radical stress on glycocalyx [56]. Additionally, antithrombin III, a physical inhibitor of serine proteases such as thrombin and elastase, which inhibits coagulation abnormalities, has also been reported to protect ECs by reducing susceptibility to enzymatic attack [57]. However, our new findings on another anticoagulant drug, rivaroxaban, another anticoagulant drug, are quite particularly significant, as antithrombin is typically administered with heparin or heparinoids, a combination unfavorable for the binding of antithrombin to the VE glycocalyx.

In our study, rivaroxaban was shown to be a safe agent for HUVECs at concentrations up to 10 μg/mL, protecting the EC glycocalyx from damage induced by H_2O_2. This protective effect was accompanied by an improvement in cell viability. Control over the activity of the PI3K/AKT pathway appears to be crucial in these effects; PI3K-dependent EC viability, apoptosis, and endothelial glycocalyx synthesis-related proteins were elevated in our observations, consistent with former reports [58–60]. According to a previous study, edoxaban, another FXa inhibitor, stabilizes ECs, counteracts the antiangiogenic effects of FXa, and has remarkable anti-inflammatory action, preventing peripheral blood mononuclear cell adhesion and transmigration through the endothelium. Its main molecular mechanism seems to be the regulation of the PAR1-2/PI3K/ nuclear-factor-kappa B pathway under the stimulation of FXa [61]. In agreement with this study, in our experiments, the inhibition of PI3K by a specific pharmacological inhibitor (LY294002) underscored the important role of rivaroxaban in improving glycocalyx damage and cell viability. Furthermore, interestingly, we were able to demonstrate the direct protective effect of rivaroxaban without the addition of exogenous FXa. This could be explained by the physiological function of human ECs, which produce their own coagulation factors that can activate cell surface FX without the addition of exogenous proteins or phospholipids (Fig. 1b) [38].

The non-hemostatic functions of FXa are largely dependent on signaling pathways mediated by PARs [62]. PARs are a family of related G-protein-coupled receptors that are activated upon cleavage of their extracellular domain [63]. For PAR1, PAR2, and PAR4, these cleavage sites generate various tethered ligands, which can alter cellular responses [64]. FXa promotes conversion from prothrombin to thrombin and subsequent activation of PAR1 [65]. In addition to promoting thrombin activation, FXa can directly signal through PAR1 and PAR2 and may vary in many different pathophysiological states [66]. According to previous studies, both PAR1 and PAR2 promote activation of the PI3K/Akt pathway, which is related to a wide range of cellular biological processes [67, 68]. These receptors play significant roles in modulating inflammatory responses by influencing cytokine production and leukocyte recruitment and are integral to maintaining endothelial barrier integrity. Activation of PAR1 and PAR2 can lead to endothelial barrier disruption [69, 70], which facilitates immune cell transmigration during inflammatory responses [71]. In the context of therapeutic intervention, the use of specific antagonists such as SCH79797 for PAR1 and AZ3451 for PAR2 has been shown to mitigate these effects. SCH79797 acts as a competitive antagonist, directly blocking the binding of agonists like thrombin to PAR1, thereby inhibiting the downstream signaling cascades that lead to inflammation and barrier disruption [72]. Alternatively, AZ3451 represents a novel approach to receptor inhibition through its allosteric mechanism of action. Unlike competitive antagonists, AZ3451 binds to a site distinct from the ligand-binding domain on PAR2, inducing a conformational change that reduces the receptor's ability to engage in its natural signaling pathways [73]. This allosteric inhibition is particularly valuable because it can potentially modulate the receptor's function in a more subtle and regulated manner, potentially reducing side effects associated with complete receptor blockade.

In the present study, the inhibition of PAR1+PAR2 using their specific antagonists (SCH79797 and AZ3451) improved H_2O_2-induced cell damage, although PAR1 or PAR2 inhibition alone was not able to rescue cell viability. This is consistent with the functional mechanism of rivaroxaban and has implications, suggesting that FXa inhibitors can be more effective than selective PAR1 inhibitors, such as thrombin inhibitors, in terms of cellular protection. Moreover, as AZ3451 is an allosteric antagonist, our observations indicate that subtle modulations of PAR2 activity (rather than complete inhibition) are sufficient to protect against oxidative damage induced by H_2O_2.

IQGAP1 is a VEGFR-2 binding protein that interacts with actin, cadherin, and β-catenin, thereby regulating cell functions [74]. Moreover, it has been shown that IQGAP1 and α-catenin compete for binding to β-catenin and that IQGAP1 dissociates α-catenin from the β-catenin-α-catenin complex in vitro [75]. In line with earlier research, our study demonstrated that IQGAP1 was localized at the cell membrane with VE-cadherin in unstimulated ECs, possibly stabilizing the cadherin-catenins complex, which results in strong cell-cell adhesion [74]. In contrast, oxidative stress induced the loosening of stable cell-cell contact, suggesting that IQGAP1 interacts with β-catenin, dissociating α-catenin from the cadherin-catenins complex, which may lead to weak cell-cell adhesion. From our findings, we propose a model in which rivaroxaban prevents IQGAP1 from interacting with β-catenin and maintains strong cell-cell adhesion under oxidative stress. Interestingly, when rivaroxaban was added to unstimulated ECs, IQGAP1 translocated to the nucleus, where the complex may modulate the transcription of several genes [76, 77]. The underlying mechanism remains to be investigated. IQGAP1 functions as a scaffold, assembling crucial components of PI3K-Akt pathway and works as a key controlling cell growth or survival [78]. In HUVECs, it has been shown that statin preparations repairs lipopolysaccharide-stimulated

endothelial injury through the PI3K/Akt/IQGAP1 pathway activity and platelet-derived growth factor receptor β [79]. In summary, our results suggest that PAR1-2/PI3K/IQGAP1 pathway is an important regulator in the protective process induced by H_2O_2 injury.

The glycocalyx is also able to directly interact with different plasma proteins and circulating cells, regulating inflammation and coagulation [27]. For instance, an intact glycocalyx acts as a barrier to leukocyte adhesion. Increased adhesion of leukocytes is observed only upon degradation of the glycocalyx. On the other hand, coagulation FXa is known to stimulate inflammatory changes, including MCP1-expression or IL-8-release, in ECs and leukocyte under conditions of excessive oxidative stress [80–82]. Our study reveals a possibility that rivaroxaban may also protect against monocyte EC-adhesion by reducing the production of inflammation mediators, and that the regulation of glycocalyx can certainly be the key to this mechanism.

In this study, we have elaborated on the fundamental role of the endothelial glycocalyx in vascular health, underlining its composition and function in maintaining endothelial integrity and mediating cellular responses. However, it is crucial to consider the limitations inherent in our experimental model which utilizes a static, monolayer setup of ECs. In vivo, the endothelial glycocalyx is subjected to constant hemodynamic forces, particularly shear stress from blood flow, which significantly influences its structure, function, and the cellular behavior of ECs. Shear stress has been shown to affect the production of critical components of the glycocalyx and modulates its protective functions against oxidative stress and inflammation [83]. The absence of flow conditions in our static cell culture model means that the dynamic interactions between blood components and the glycocalyx, crucial for its physiological and pathological responses, are not replicated. This limitation could affect the generalizability of our findings to in vivo conditions, where fluid shear stress plays a pivotal role in glycocalyx maintenance and function. In addition, the use of ECs cultured in a monolayer does not fully capture the complex three-dimensional architecture and multicellular interactions present in blood vessels. In vivo, ECs interact not only with each other but also with other types of cells such as smooth muscle cells and pericytes, which contribute to the overall vascular function and stability. The simplified model of a cell monolayer might not adequately reflect these interactions or the spatial organization of the glycocalyx and its associated cellular components. Given these limitations, while our findings provide valuable insights into the protective effects of interventions like rivaroxaban on the endothelial glycocalyx under oxidative conditions, caution must be exercised when extrapolating these results to more complex in vivo settings. Future studies could benefit from incorporating dynamic flow models, such as microfluidic systems or organ-on-a-chip technologies, which allow the simulation of blood flow and its mechanical effects on ECs. Additionally, employing advanced 3D culture systems or co-culture models could help in better mimicking the in vivo vascular environment, thereby enhancing the physiological relevance of the findings [84].

Here, we demonstrate a new insight into an in vitro model of oxidative stress-induced vascular damage in which a single use of rivaroxaban may have a protective effect on ECs. This suggests potential applications beyond its primarily use in thrombosis prevention, including benefits in managing or preventing conditions like atherosclerosis or vascular inflammation where glycocalyx degradation plays a key role. Furthermore, it could improve outcomes in conditions of oxidative stress such as diabetes, sepsis, or ischemia/reperfusion injury. These findings could also stimulate further research into new therapeutic strategies that target glycocalyx preservation.

Conclusions

Rivaroxaban protects against oxidative stress-induced glycocalyx damage via IQGAP1 scaffolding to maintain vascular permeability, through the PI3K/Akt cell survival signaling pathway.

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Statement of Ethics

HUVECs used in our experiments were purchased from Promo Cell, and no animal experiment or human's tissue were included in our study. According to the "3rd Scope" of the Ethical Guidelines for Medical and Biological Research Involving Human Subjects, these guidelines do not apply to specimens and information that have already been established as having academic value, are widely used for research purposes, and are generally available. Therefore, ethical approval was not required under national guidelines or the guidelines of the Clinical Research Review Board of the Kitasato Institute.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

L.K. conceived, performed all the experimental work, and wrote the manuscript with input from all authors. T.I., Y.M., T.H., M.S., N.M., and R.K. provided technical assistance on experiments, J.O. supervised the quantitative work. Y.I., R.K., M.Y.-T., and F.O. supervised the work gave their expertise, revised the manuscript. J.A. directed the project.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further enquiries can be directed to the corresponding author.

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