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Full Length Article

# Alteration in endothelial permeability occurs in response to the activation of PAR2 by factor Xa but not directly by the TF-factor VIIa complex

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ABSTRACT

Alterations in the endothelial permeability occur in response to the activation of coagulation mechanisms in order to control clot formation. The activation of the protease activated receptors (PAR) can induce signals that regulate such cellular responses. PAR2 is a target for the coagulation factor Xa (fXa) and tissue factor-factor VIIa (TF-fVIIa) complex. By measuring the permeability of dextran blue across endothelial monolayer, we examined the mechanisms linking coagulation and endothelial permeability. Activation of PAR2 using the agonist peptide (PAR2-AP) resulted in increased permeability across the monolayer and was comparable to that obtained with VEGF at 60 min. Incubation of cells with activated factor Xa (fXa) resulted in an initial decrease in permeability by 30 min, but then significantly increased at 60 min. These responses required fXa activity, and were abrogated by incubation of the cells with a PAR2-blocking antibody (SAM11). Activation of PAR2 alone, or inhibition of PAR1, abrogated the initial reduction in permeability. Additionally, inclusion of Rivaroxaban (0.6 μg/ml) significantly inhibited the response to fXa. Finally, incubation of the endothelial monolayers up to 2 h with TF-containing microvesicles derived from MDA-MB-231 cells, in the presence or absence of fVIIa, did not influence the permeability across the monolayers. In conclusion, fXa but not TF-fVIIa is a noteworthy mediator of endothelial permeability. The rapid initial decrease in permeability requires PAR2 and PAR1 which may act to constrain bleeding. The longer-term response is mediated by PAR2 with increased permeability, presumably to enhance clot formation at the site of damage.

## 1. Introduction

Under physiological conditions, the endothelial layer acts as one of the regulators of the haemostatic mechanism, acting as a barrier between the blood and the underlying thrombogenic tissue. However, this barrier is dynamic and is regulated to conform to the requirements of the local vasculature. Alterations in endothelial permeability are essential in the maintenance of the correct homeostatic functions but also, are often central in the pathogenesis of inflammatory and vascular disorders. Following injury, the responses by the vasculature limits blood flow and then allows seepage into the sub-endothelial layer. Protease activated receptors are G-protein coupled receptors which act as sensors for the presence of active proteases, in particular those that are involved in the coagulation mechanism [1,2]. The endothelial cells express PAR1 and PAR2 which mediate numerous outcomes that include changes to endothelial permeability [3–5]. PAR2 signalling is induced following the proteolysis by coagulation factor Xa (fXa), tissue factor-factor VIIa (TF-fVIIa) complex as well as by trypsin I, II and IV, tryptase, acrosin, granzyme A and kallikrein 2, 4, 6 and 14, in a canonical manner, and by elastase, proteinase 3 and cathepsins G and S in a non-canonical system [6,7]. The activation of PAR2 has also been associated with inflammatory responses during chronic disorders [2,8] and suggested as a cause of vascular complications associated with various diseases [4,9].

Endothelial cells are connected to each other by a complex set of junction proteins [10–13]. Under normal conditions, inter-endothelial junctions open to permit the passage of molecules and surveillance cells in a dynamic and size-selective manner [14–16]. Moreover, a number of inflammatory agents including thrombin and VEGF, are able to stretch out these junctions permitting contact between plasma and the sub-endothelial layer [17–19]. It has been shown that endothelial cells respond to coagulation by altering the vascular permeability [20].

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However, little is known of the endothelial responses under conditions of coagulation, when the proteases are simultaneously generated. Furthermore, the overall response is likely to also be dependent on the magnitude of coagulation activation, which in turn would be dependent on the extent of the injury incurred. The role of PAR2 in the alteration of vascular permeability has not been defined. Current reports equally ascribe the activation of PAR2 signalling to increases in endothelial permeability [8,21,22], and refute such a response [23,24]. In addition, it has been suggested that PAR2 acts in conjunction with PAR1 to desensitize subsequent cellular responses to thrombin [5]. In this study we have examined the ability of fXa and TF-fVIIa to influence the permeability of endothelial monolayers. In addition, by inhibiting fXa activity and blocking PAR2 activation, the role of these proteins in the regulation of endothelial permeability is further clarified.

## 2. Material and methods

### 2.1. Cell culture and endothelial-barrier permeability assay

Human coronary artery primary endothelial cells (HCAEC) and human dermal blood-microvascular endothelial cells (HDBEC), devoid of endogenous TF were cultured in MV media containing 5% (v/v) foetal calf serum (FCS) and growth supplements (PromoCell, Heidelberg, Germany). MDA-MB-231 breast cancer cell line (ATCC, Teddington, UK) was cultured in DMEM, containing 10% (v/v) FCS.

The *in vitro* endothelial barrier assay was adapted by modification of a previously described procedure [5]. Transwell polyester membrane inserts (3.0 μm pore size; Thermo Scientific, Warrington, UK) were coated with 0.5% (w/v) gelatin (Sigma Chemical Company Ltd., Poole, UK) and incubated for 2 h prior to usage. Cells ($6 \times 10^4$) were seeded out and cultured in complete endothelial cell medium and propagated for 4 days to obtain confluent monolayers. The permeability of the monolayers was assessed under test conditions without any stimulus and found to remain uncompromised for > 2 h. On the day of the experiments, the monolayers were washed twice with phosphate buffered saline (PBS). The cells were then incubated for 1 h in with equal volumes of serum-free endothelial medium at the upper and lower chambers. Blue dextran MW $4 \times 10^4$ (Sigma) was diluted to a final concentration of 1 μg/ml in PBS was then added to the upper chamber of each Transwell. Any test-chambers with apparent leakages at the start of the experiment were excluded. The test reagents were then added to the upper chambers and incubated at 37 °C for up to 120 min. Aliquots of the media (200 μl) were removed from the bottom chamber and the absorption measured at 584 nm.

### 2.2. Preparation of test reagents

Rivaroxaban was obtained as pure compounds from Bayer (Leverkusen, Germany), dissolved in dimethyl sulfoxide (DMSO) and diluted in PBS to 4 mg/ml working stock solutions. Appropriate controls using similarly diluted DMSO were used alongside, as controls. The final concentrations of the Rivaroxaban were optimised against purified fXa beforehand to ensure maximal inhibition. Vascular endothelial growth factor (VEGF)-A (TCS Cellworks/Caltag Medsystems Ltd., Buckingham, UK) was diluted to 25 ng/ml final concentration in the base culture medium. Protease activated receptor 2-agonist peptide (PAR2-AP; SLIGKV) and PAR1-AP (TFLLR) were synthesised (Severn Biotech Ltd., Kidderminster, UK) and used at a final concentration of 20 μM [25]. Coagulation factors Xa and VIIa (Enzyme Research Laboratories, Swansea, UK) were diluted stepwise to the required concentrations (10 nM and 5 nM) in the base culture medium. Recombinant TF (Innovin thromboplastin reagent; Dade Behring, Deerfield, USA) was used at a final concentration of 1 U/ml (1.3 ng/ml). Blocking antibodies for PAR2 (SAM11) and PAR1 (ATAP2) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and were incubated with the cells at 20 μg/ml to prevent the activation of PAR2 and PAR1 respectively.

### 2.3. Preparation and quantification of the TF-containing microvesicles

MDA-MB-231 cells were propagated in 25 $cm^2$ flasks. To generate the TF-containing microvesicles, the cells were washed and adapted to DMEM serum-free medium, for 2 h. The released microvesicles were then prepared from the conditioned media by ultracentrifugation according to described procedures [26]. The functional density of the released of microvesicles was determined using the Zymuphen MP-assay kit (Hyphen BioMed/Quadratech, Epsom, UK) and the microvesicle density determined from the standards provided by the kit. The released microvesicle-associated TF antigen was measured using the Quantikine TF-ELISA kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Cell surface TF antigen was measured *in situ* using an ELISA-based procedure a previously described [27,28].

### 2.4. Chromogenic activity assays

Thrombin generation potential of the purified microvesicles was confirmed using a two-stage chromogenic thrombin generation assay as before [27]. The activity of fXa was measured using a chromogenic fXa substrate (0.2 mM; Hyphen).

### 2.5. Statistical analysis

All data represent the calculated mean values from the number of experiments stated in each figure legend ± the calculated standard error of the mean. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc. Chicago, USA). Significance was determined using one-way ANOVA (analysis of variance) and Tukey's honesty significance test or where appropriate, by paired *t*-test.

## 3. Results

To ensure that the endothelial monolayers were not affected for the duration of the assay by the lack of serum, the presence of blue dextran or the inclusion of DMSO, initially the Transwell-monolayers were monitored over 2 h and confirmed to remain intact (not shown). Incubation of the monolayer without any stimulus resulted a gradual increase in the transfer of dextran blue as expected, but this was not significant over the period of analysis (Fig. 1) and therefore deemed to be a stable model.

### 3.1. Activation of PAR2 by fXa alters the permeability across the endothelial monolayer

The activation of both HCAEC and HDBEC using PAR2-AP resulted in increased permeability within 60 min but was less than that induced by VEGF (Fig. 1). No further increase in the permeability was detected after this time point. Furthermore, incubation of cells with PAR1-AP did not result in any significant change in the permeability of endothelial monolayer which in agreement with some [5], but contradictory to other reports [24]. Incubation of HCAEC and HDBEC monolayers with fXa (10 nM) prevented the change in the permeability across the monolayers and therefore, resulted in a relative decrease when compared to the untreated sample at the same time point (Fig. 2). This was replaced by a significant increase in the leakage of blue dextran (80% and 92% increase) across HCAEC and HDBEC monolayers respectively, at 60 min post-addition of fXa. Incubation of endothelial monolayers with a lower concentration of fXa (5 nM) did not result in the initial reduction in permeability at 30 min and the ensuing increase in permeability was not significant.

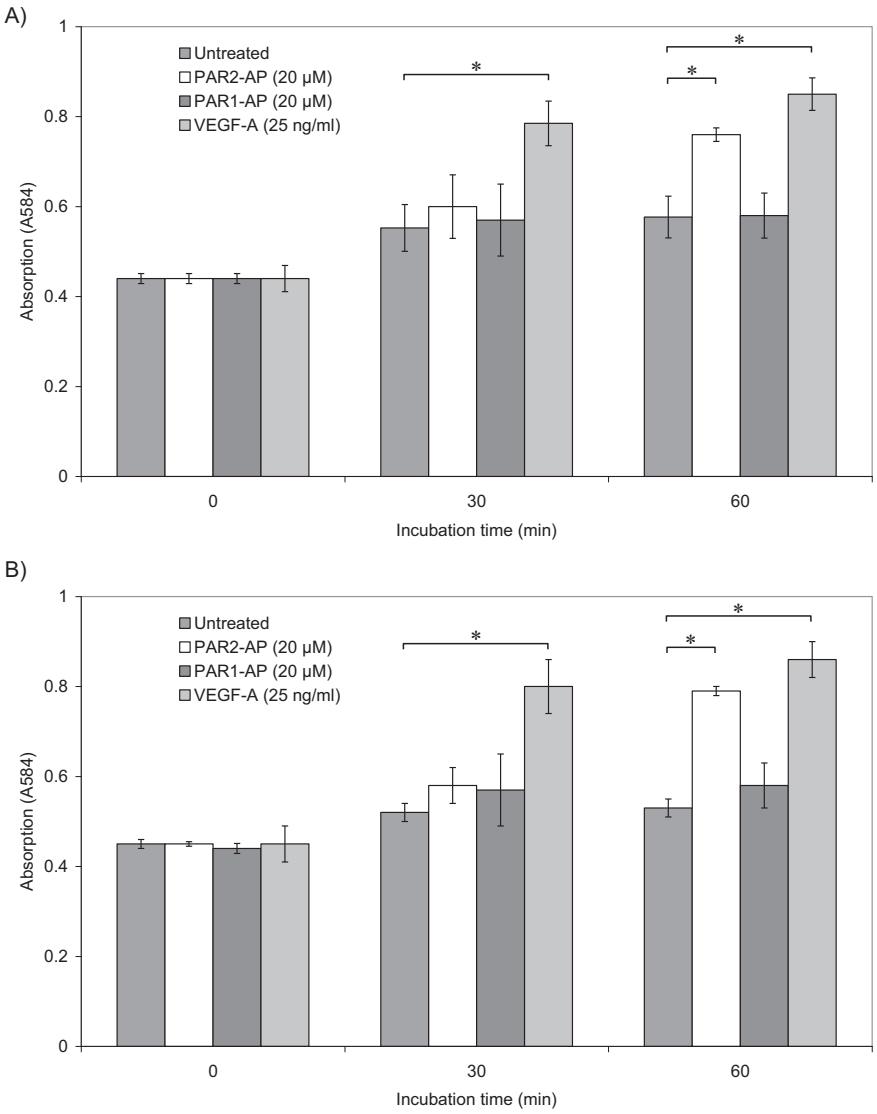


**Fig. 1.** Increase in endothelial permeability on PAR2 activation. Monolayers of HCAEC (A) and HDBEC (B) were prepared by culturing the cells ($6 \times 10^4$) in gelatin-coated Transwell chambers for 4 days. The monolayers were washed twice with PBS and adapted to serum-free endothelial medium. Blue dextran was added to a final concentration of 1 μg/ml to the upper chamber of each Transwell. Sets of cells were activated with PAR2-AP (20 μM), PAR1-AP (20 μM) or VEGF-A (25 ng/ml) added to the upper chambers. The samples were incubated at 37 °C for up to 60 min. Aliquots of the media (200 μl) were removed from the bottom chamber and the absorption measured at 584 nm measured. (n = 4; * = $p < 0.05$).

### 3.2. The induction of endothelial permeability requires fXa proteolytic activity

Rivaroxaban is direct oral anticoagulant which function through binding and directly inhibiting fXa. To ensure complete inhibition of fXa, samples of fXa were pre-incubated with therapeutic and sub-therapeutic concentrations of Rivaroxaban (0.6 and 0.06 μg/ml) prior to measuring the residual fXa activity using a fXa-chromogenic substrate (Fig. 3). In order to examine the requirement for the proteolytic function of fXa, endothelial monolayers were incubated with fXa (10 nM) and also in the presence of Rivaroxaban (0.06 and 0.6 μg/ml). Inclusion of Rivaroxaban (0.6 μg/ml) significantly inhibited the response of the endothelial monolayer to fXa (92% inhibition) (Fig. 4A). Rivaroxaban also prevented the initial reduction in permeability at 30 min (Fig. 4B).

### 3.3. The induction of endothelial permeability is mediated through PAR2 activation

To determine if the observed changes in permeability were as a consequence of the proteolytic activation of PAR2 by fXa, sets of HCAEC and HDBEC were pre-incubated with blocking antibodies to PAR2 and PAR1. Inhibition of PAR2 using SAM11 antibody prior to addition of fXa (10 nM) abrogated all changes in permeability of the monolayer in response to fXa (Fig. 5). Activation of cells with fXa in the presence of PAR1 blocking antibody (ATAP2) resulted in the omission of the initial reduction in permeability. However, the increase in permeability at 60 min was not significantly altered when compared to fXa alone.

### 3.4. Factor Xa but not TF-fVIIa complex is capable of inducing endothelial permeability

In addition to fXa, the TF-fVIIa complex is known to be capable of activating PAR2 directly. Therefore, the possibility that TF-containing microvesicles may also be capable of increasing the endothelial permeability through PAR2 activation was explored. TF-containing microvesicles were isolated from MDA-MB-231 cells and confirmed to posses significant thrombin generation potential at 10 nM. Thereafter, endothelial monolayers were incubated with TF-containing microvesicles (10 nM final concentration), or alternatively with recombinant TF (1 U/ml), in the presence or absence of additional fVIIa (5 nM). Incubation of HCAEC for 60 or 120 min with TF-containing microvesicles, or recombinant TF was largely ineffective in increasing the permeability of the monolayers and remained unaltered on inclusion of fVIIa (Fig. 6A). Finally, the inclusion of both fXa and fVIIa with either the microvesicles or recombinant TF did not enhance the permeability of the endothelial monolayers, beyond that observed with fXa alone (Fig. 6B).

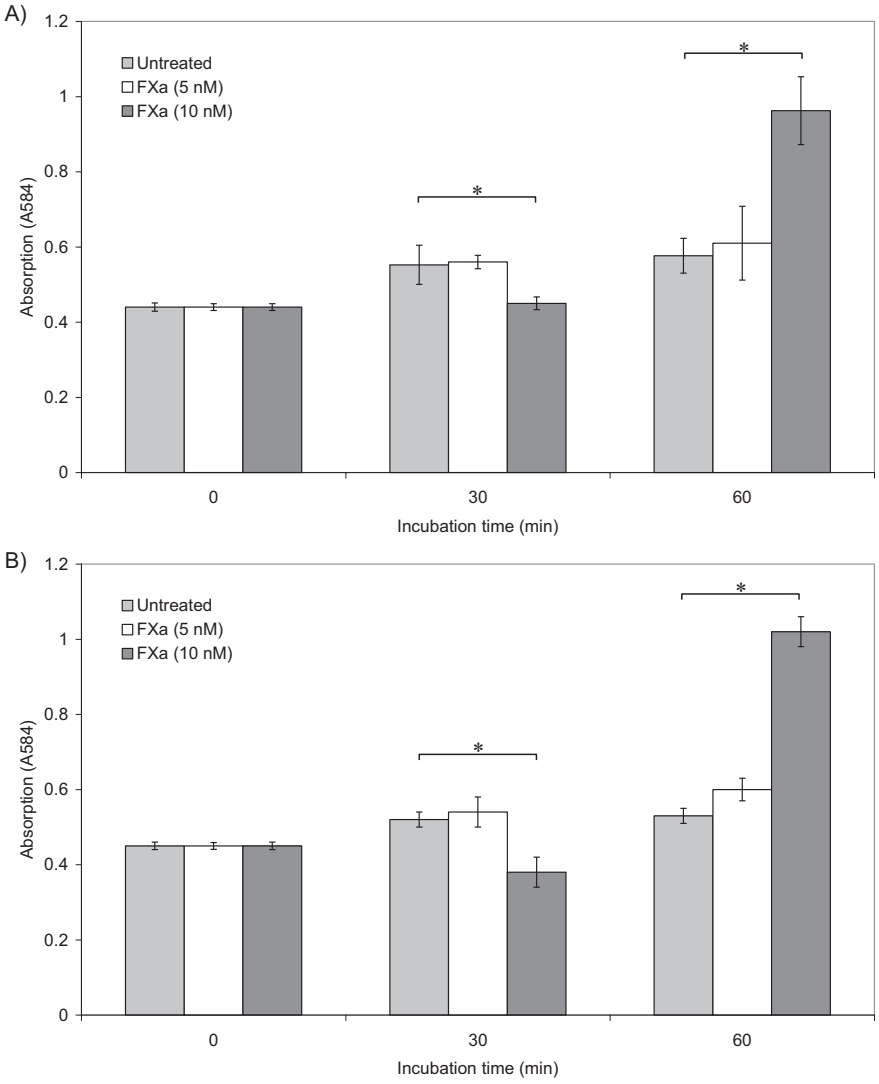


**Fig. 2.** Increase in endothelial permeability in response to fXa. Monolayers of HCAEC (A) and HDBEC (B) were prepared by culturing the cells ($6 \times 10^4$) in gelatin-coated Transwell chambers for 4 days. The monolayers were washed twice with PBS and adapted to serum-free endothelial medium. Blue dextran was added to a final concentration of 1 ng/ml to the upper chamber of each Transwell. Sets of cells were activated with factor Xa (0–10 nM) added to the upper chambers. The samples were incubated at 37 °C for up to 60 min. Aliquots of the media (200 μl) were removed from the bottom chamber and the absorption measured at 584 nm measured. (n = 4; * = $p < 0.05$).

## 4. Discussion

The ability of the vasculature to determine the nature and magnitude of a stimulus is essential for the appropriate and proportionate response. The vascular response to injury include rapid changes to blood flow to reduce the loss of blood, followed by increased contact with the sub-endothelial layer, in order for the formation of a stable and effective clot [29–32]. In agreement with this hypothesis the incubation of the endothelial monolayers with fXa resulted in an initial reduction in permeability followed by significantly higher levels of leakage across

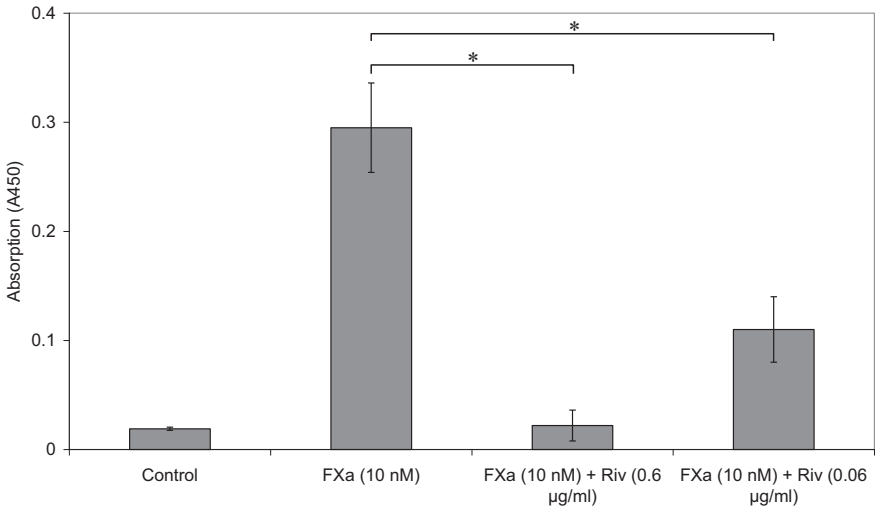


**Fig. 3.** Therapeutic concentrations of Rivaroxaban fully inhibit the activity of fXa. Samples of fXa (10 nM) were incubated with Rivaroxaban (0.06 and 0.6 μg/ml) or DMSO vehicle. The samples were incubated in the presence of a fXa substrate (0.2 mM) and the fXa protease activity evaluated by measuring the absorptions at 405 nm. (n = 3; * = $p < 0.05$).

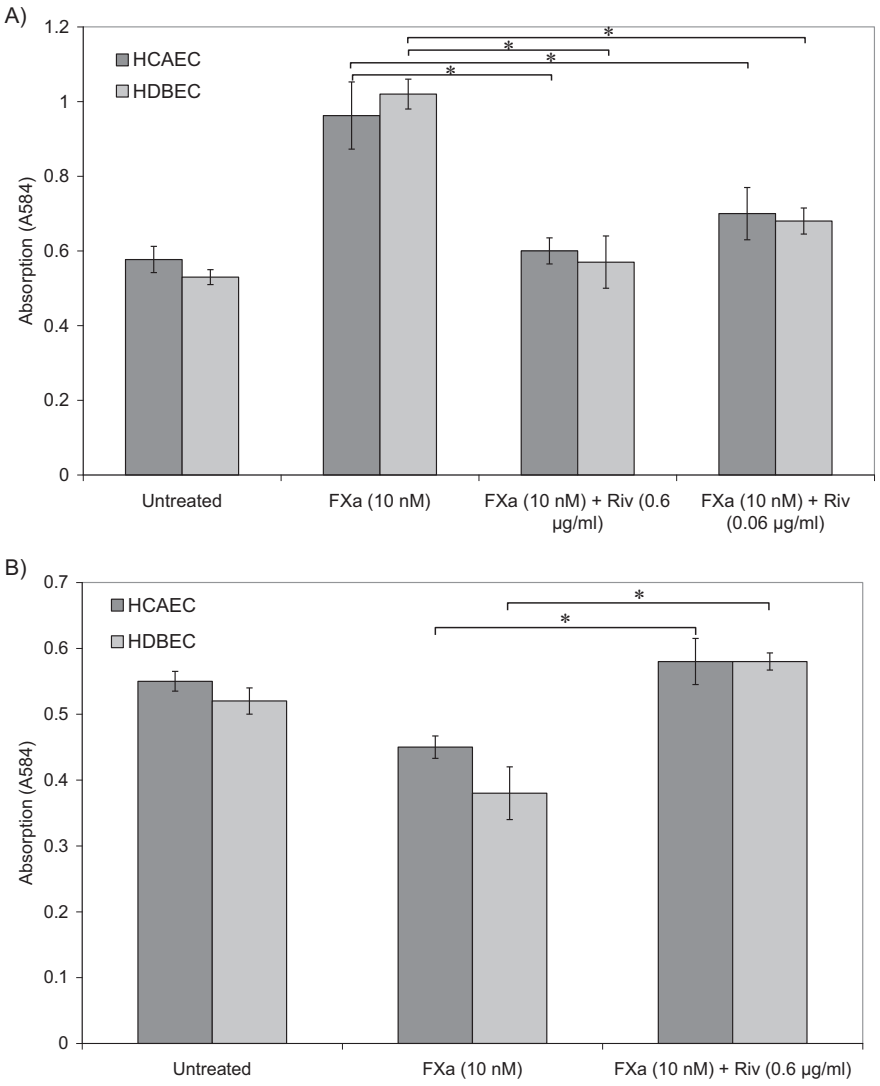


**Fig. 4.** The increase in endothelial permeability is dependent on fXa protease activity. Monolayers of HCAEC (A) and HDBEC (B) were prepared by culturing the cells ($6 \times 10^4$) in gelatin-coated Transwell chambers for 4 days. The monolayers were washed twice with PBS and adapted to serum-free endothelial medium. Blue dextran was added to a final concentration of 1 µg/ml to the upper chamber of each Transwell. Sets of cells were activated with factor Xa (10 nM) in the presence of Rivaroxaban (0.06 and 0.6 µg/ml) or DMSO vehicle. The samples were incubated at 37 °C and the effect of Rivaroxaban was also monitored at 60 min (A) and also monitored at 30 min (B). Aliquots of the media (200 µl) were removed from the bottom chamber and the absorption measured at 584 nm measured. (n = 4; * = $p < 0.05$).

to the lower chamber. Interestingly, incubation of endothelial monolayers with sub-optimal levels of fXa (5 nM) did not produce any response while at 10 nM, the enzyme produced full response in the cells. The magnitude of increased permeability at 60 min, following activation with fXa (10 nM) was higher than that obtained with PAR2-AP. Furthermore, the response to PAR2-AP did not produce the initial tightening of the endothelial layer at 30 min. Activation of fX within the blood is initiated by the TF-fVIIa complex and therefore the relatively lower amounts of fXa generated by the extrinsic pathway may be incapable of causing vascular permeability. Conversely, full activation of the coagulation involving the intrinsic pathway can convert a large quantity of the available fX at the locality of the injury. Therefore, it is plausible that under these conditions the adaptations in the permeability of the endothelial layer to allow contact between plasma and the sub-endothelial layer, becomes an essential step in the completion and strengthening of the clot. This mechanism appears to be dependent on the proteolytic activity of fXa and was significantly diminished in the presence of Rivaroxaban. As stated above, while some studies report that the activation of PAR2 signalling promotes endothelial permeability [8,21,22], others did not find a response [23,24]. Furthermore, Feistritzer and Riewald [5] suggested that PAR1 and PAR2 act in conjunction to desensitize endothelial response to further activation. Our data indicate that the activation of PAR2 appears to be a major inducer of permeability and may participate in the original reduction endothelial tightness. A recent report has shown that the activation of PAR2 can disrupt cellular tight junctions through the activation of p38 MAPK alone [33]. However, concurrent activation of ERK1/2 and Akt pathways in response to PAR2 activation may occur independently [33,34]. Furthermore, some PAR2 signalling appears to be dose dependent even when using the activating peptide [35]. These data are in accord with our finding that the induction of PAR2 using the activating peptide results in the activation of p38 MAPK the magnitude of which can further be modulated by other factors including the presence of TF [36]. Moreover, the activation of PAR2 alone using the agonist peptide did not produce the initial tightening of the endothelial layer at 30 min. Consequently, PAR1 appears to be an essential contributor to the initial reduction in permeability. Therefore, the responses of PAR2, in conjunction with PAR1 appear to constitute a means of converting the signal arising from fXa into the converse but canonical and time-dependent modifications in endothelial permeability. It has been recognised that while PAR2 constitutes the main receptor for fXa induced signalling, PAR1 also acts as a minor target for fXa [37] and may be involved in fXa signalling [38]. The protective influence of PAR1 [5,39,40] in thrombin-mediated signalling, on endothelial function has been documented [4,5]. Moreover, the ability of PAR1 to induce endothelial permeability is known [41]. However, thrombin appears to influence vascular permeability through multiple mechanisms including direct signalling, by influencing other cells through the nitric oxide-mediated pathways. Moreover, the outcomes of response to thrombin appear to be both cell-specific and target-selective, affecting

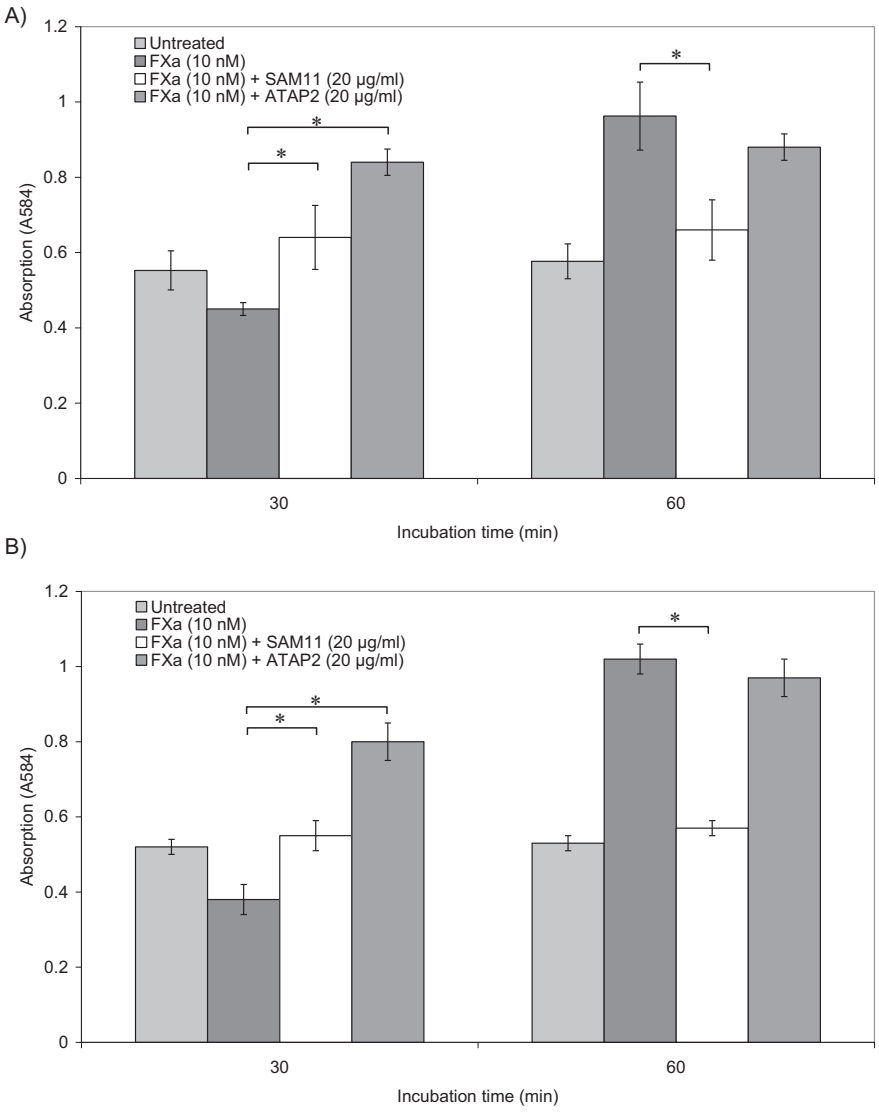


**Fig. 5.** The increase in endothelial permeability is mediated through PAR2 activation. Monolayers of HCAEC (A) and HDBEC (B) were prepared by culturing the cells ($6 \times 10^4$) in gelatin-coated Transwell chambers for 4 days. The monolayers were washed twice with PBS and adapted to serum-free endothelial medium in the presence or absence of PAR2 and PAR1 blocking antibodies (SAM11 and ATAP2 respectively). Blue dextran was added to a final concentration of 1 μg/ml to the upper chamber of each Transwell. Sets of cells were activated with factor Xa (10 nM) added to the upper chambers. The samples were incubated at 37 °C for up to 60 min. Aliquots of the media (200 μl) were removed from the bottom chamber and the absorption measured at 584 nm measured. (n = 5; * = $p < 0.05$).

various different proteins within the cellular tight junction [42]. In addition, some of the differences in the outcomes appear to arise from the requirement for cell-surface effectors such as APC or PAR3 [4,43] although some reports dispute a direct role for PAR3 in the response to PAR1 or the expression of PAR4 on some endothelial cells [44]. Finally, it has been suggested that the magnitude of activation of PAR1 is amplified by the proteolytic action of thrombin, in comparison to for example by PAR1-activating peptide [45,46]. Therefore, we are not able to present a conclusive explanation for the lack of alterations in permeability in response to PAR1-activating peptide. Additionally, the focus of our study has been the role of fXa as a first messenger in regulating the induction of endothelial permeably in response to the activation of coagulation. Consequently, the discussions on the multiple roles of thrombin and the magnitude of PAR1 activation, together with the multiple second messengers and pathways involved in PAR signalling [3,4] is beyond the scope of our study. Consequently, the outcome of PAR1 activation was not further pursued in this study. However, in addition to the contribution from PAR1, co-operative signalling from other receptors such as EPCR [53,54] may also contribute to PAR2 signalling and require clarification.

The initial reduction and the subsequent increase in permeability proposes a regulatory process to control bleeding. However, following repeated injury, or during long-term presence of the inflammatory stimuli, these mechanisms may become defective and even detrimental. The expression of TF is a hallmark of chronic inflammatory conditions but TF-fVIIa complex is not always fully active and can exist in an encrypted or partially active format [47–52]. In our experiments, either recombinant TF, or TF contained within microvesicles derived from a cell line did not affect the endothelial permeability. Intriguingly, the inclusion of fVIIa with the TF also was not capable of eliciting similar responses to fXa. Previously we showed that TF-containing microvesicles from MDA-MB-231 cells begin to be assimilated within the endothelial cell membrane within 30 min of incubation [55]. To ensure sufficient intervals cell permeability was analysed at 60 and 120 min following induction. This finding suggest that the presence of TF *per se* is not sufficient to induce such alterations in permeability which in turn may escalate into thrombus formation [56,57]. Moreover, this observation questions the role of plasma seepage arising from endothelial permeability, as the main cause of distal thromboembolism, for example in cancer patients who have detectable amounts of circulating TF-containing microvesicles. However, the possibility of the involvement of a ternary complex TF-fVIIa-fXa is not dismissed and it is feasible that once sufficient fXa is generated by the coagulation mechanism, the induction of permeability may be enhanced by TF-fVIIa-fXa complex formation. Regardless, the use of anti-fXa agents such as direct oral anticoagulants may be effective in limiting the vascular permeability and oedema formation.

The crosstalk between the coagulation mechanism and the

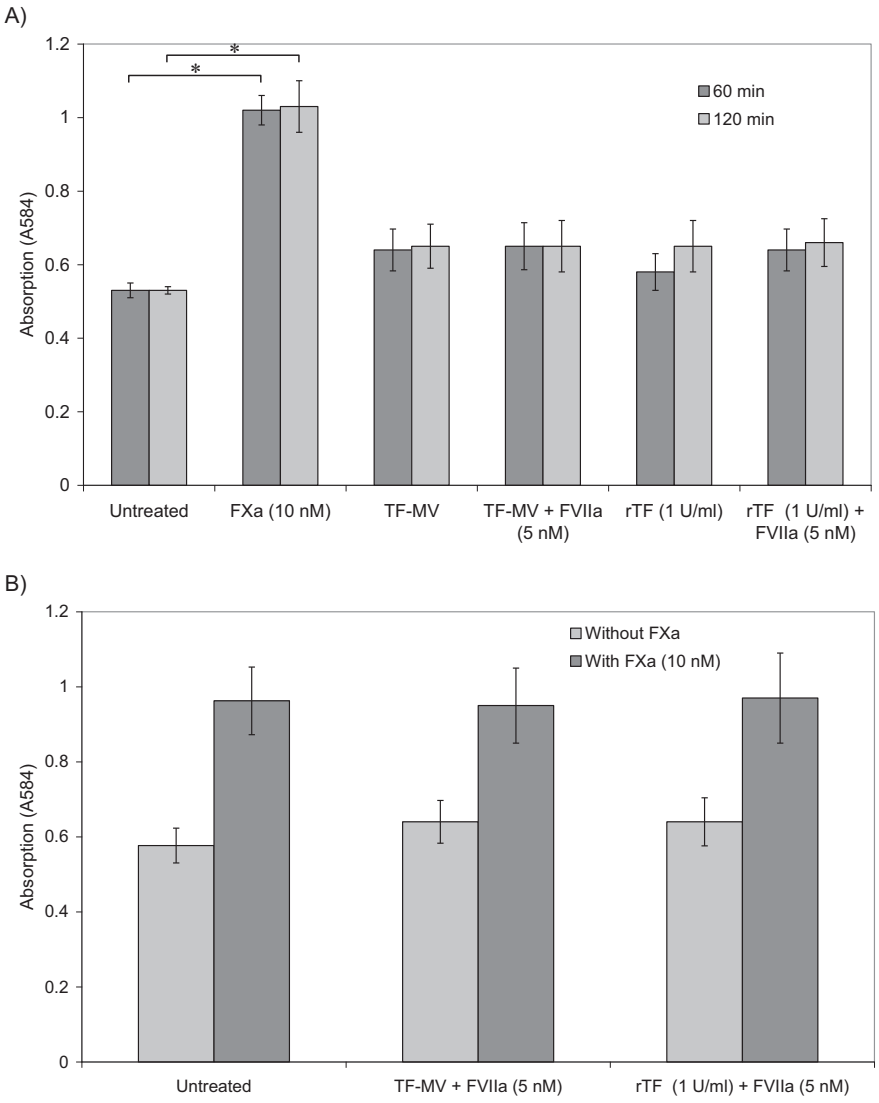


**Fig. 6.** PAR2-mediated increase in endothelial permeability occurs in response to fXa but not TF-fVIIa. Monolayers of HCAEC were prepared by culturing the cells ($6 \times 10^4$) in gelatin-coated Transwell chambers for 4 days. The monolayers were washed twice with PBS and adapted to serum-free endothelial medium. Blue dextran was added to a final concentration of 1 ng/ml to the upper chamber of each Transwell. A) Sets of cells were incubated with purified TF-containing microvesicles or with recombinant TF (1 U/ml), in the presence or absence of fVIIa (5 nM), and compared to treatment with fXa (10 nM) alone. The samples were incubated at 37 °C and examined at 60 or 120 min. Aliquots of the media (200 μl) were removed from the bottom chamber and the absorption measured at 584 nm measured. (n = 3; * = $p < 0.05$). B) Sets of cells were incubated with purified TF-containing microvesicles or with recombinant TF (1 U/ml), in the presence of fVIIa (5 nM), and in the presence or absence of fXa (10 nM). The samples were incubated at 37 °C for up to 60 min. Aliquots of the media (200 μl) were removed from the bottom chamber and the absorption measured at 584 nm measured. (n = 3).

vasculature ensures correct haemostasis following injury. Among the components involved in this communication is PAR2 which responds to generated proteases. This study indicates that the activation of PAR2 by the proteolytic action of fXa but not TF-fVIIa complex, mediates endothelial responses that initially result in the reduction of vascular permeability, and subsequently promote increased contact between blood plasma and the sub-endothelial layer.

## Authors' contributions

The study was designed by AM, MC and CE, and the experimental work carried out by NB and SF. The data were evaluated by NB, AM, MJ and CE and the manuscript was prepared by NB and CE.

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