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Our recent studies have demonstrated that the Gla domain-dependent interaction of protein C with endothelial protein C receptor (EPCR) leads to dissociation of the receptor from caveolin-1 and recruitment of PAR-1 to a protective signaling pathway. Thus, the activation of PAR-1 by either thrombin or PAR-1 agonist peptide elicited a barrier-protective response in which endothelial cells were preincubated with protein C. In this study, we examined whether other vitamin K-dependent coagulation protease zymogens can modulate PAR-dependent signaling responses in endothelial cells. We discovered that the activation of both PAR-1 and PAR-2 in endothelial cells pretreated with factor FX (FX)-S195A, but not other procoagulant protease zymogens, also results in initiation of protective intracellular responses. Interestingly, similar to protein C, FX interaction with endothelial cells leads to dissociation of EPCR from caveolin-1 and recruitment of PAR-1 to a protective pathway. Furthermore, it was revealed that, FX activated by factor VIIa on tissue factor-bearing endothelial cells also initiates protective signaling responses through the activation of PAR-2 independent of EPCR mobilization. All results could be recapitulated by the receptor agonist peptides to both PAR-1 and PAR-2. These results suggest that a cross-talk between EPCR and an unknown FX/FXa receptor, which does not require interaction with the Gla domain of FX, results in protective signaling pathways in endothelial cells.

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vate protease-activated receptors (PAR-1, PAR-2, and PAR-4) to initiate both proinflammatory and anti-inflammatory responses (12, 13, 18). For instance, FVIIa, by itself or in complex with TF, has been reported to initiate proinflammatory responses through the cleavage of PAR-2 on endothelial and other cell types (19). PAR-2 appears not to be activated by thrombin, however, FXa is thought to activate this receptor (13, 20). Furthermore, it has been demonstrated that the TF-FVIIa complex can indirectly signal through both PAR-1 and PAR-2 by activating FX to FXa on endothelial cells (18–20). In a recent report, it was demonstrated that FXa, through the activation of both PAR-1 and PAR-2 can initiate a potent barrier-protective effect in response to proinflammatory stimuli in activated human umbilical vein endothelial cells (HUVECs) (20). Other studies have assigned a proinflammatory role for FXa in similar in vitro cellular models (21–23). The basis for the discrepancy in results between different studies is not known.

In light of the observation that the occupancy of EPCR by the Gla domain of protein C can change the PAR-1-dependent signaling specificity of thrombin from a barrier-disruptive to a barrier-protective effect (15), we undertook the current study to determine whether the interaction of other coagulation zymogens with endothelial cell surface receptors can play a similar role in modulating the signaling specificity of PARs. We discovered that, in addition to protein C, the preincubation of endothelial cells with FX, but not with prothrombin, FVII, or FIX, also switches the signaling specificity of both PAR-1 and PAR-2 from a barrier-disruptive to a barrier-protective effect. Interestingly, in the case of signaling via PAR-1, the mechanism of this effect was found to be also mediated through membrane mobilization of EPCR because pre-treatment of endothelial cells with FX-S195A led to dissociation of EPCR from caveolin-1, without a requirement for the occupancy of EPCR. The Gla domain of FX is not involved in this process because the Gla domainless mutant of FX also elicited the same response. Further studies revealed that FXa, whether added exogenously to HUVECs as a protease, or activated from FX by picomolar concentrations of FVIIa on cells expressing TF, initiated a potent barrier-protective response in activated endothelial cells through the activation of PAR-2.

**EXPERIMENTAL PROCEDURES**

Construction, Expression, and Purification of Recombinant Proteins—Construction and expression of the Ser-195 → Ala (FX-S195A) (chymotrypsinogen numbering (24)) and the Gla domainless mutant of FX (GDFX) in human embryonic kidney (HEK293) cells has been described (25, 26). Expression of protein C and its S195A mutant (PC-S195A) has been described (15). A protein C mutant in the Gla domainless form (GDPC) in which 6 residues (Leu-83 to Leu-88) of the loop connecting EGF1 to the EGF2 domain of FX (GDPC/FX6aa) was expressed in the same expression system and purified to homogeneity as described (27). The construction and expression of an FX mutant lacking both Gla and EGF1 domains (E2FX) has been described (25). The E2FX mutant containing Ala substitutions for residues Arg-86 and Lys-87 (E2FX-RK/AA) was expressed using the same vector system. The cDNA fragment coding for human TF lacking its cytoplasmic domain (dc-TF) (28) was subcloned to HindIII and XbaI restriction enzymes sites of the pcDNA3.1 expression vector (Invitrogen) for transient transfection to endothelial cells.

Human plasma proteins including factors VII/VIIa, X/Xa, IX, and prothrombin were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The non-blocking anti-PAR-1 (S-19) and function-blocking anti-PAR-1 (H-111) and anti-PAR-2 (SAM-11) antibodies, and siRNA specific for sphingosine 1-phosphate receptor 1 (S1P1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The function-blocking EPCR antibody (clone RCR-252) was purchased from Cell Sciences (Canton, MA). The antibody concentrations in the blocking assays were 25 μg/ml in all experiments. Anti-caveolin-1 antibody was purchased from BD Biosciences (San Jose, CA). Pertussis toxin (PTX) and methyl-β-cyclodextrin (MβCD) were purchased from Sigma. Tumor necrosis factor-α (TNF-α) was purchased from R&D Systems (Minneapolis, MN). Thrombin receptor agonist peptides (TRAP specific) for the activation of PAR-1 (TFLLRN) and PAR-2 (SLIGKV) were purchased from Bachem Bioscience (Torrance, CA). The anti-NF-κB p65 antibody detecting the endogenous levels of total NF-κB p65 and the anti-phospho-NF-κB p65 antibody detecting the activated Ser-536-phosphorylated form of NF-κB p65 were purchased from Cell Signaling Technology (Danvers, MA). The kits for monitoring the activation of RhoA and Rac1 GTPases were obtained from Cytoskeleton (Denver, CO).

**Permeability Assays**—The barrier permeability of primary HUVECs (purchased from Cambrex Bio Science Inc., Charles City, IA) or transformed HUVECs (EA.hy926 cells, kindly provided by Dr. C. Edgell from University of North Carolina at Chapel Hill, NC) in response to thrombin (5 nM for 10 min), following treatment with either APC or FXa (20 nM, 3 h), was quantitated by spectrophotometric measurement of the flux of Evan’s blue-bound albumin across the cell monolayers using a modified 2-compartment chamber model as described (15). For the function-blocking antibody treatments of the monolayers, medium was removed and antibodies were added for 30 min in serum-free medium followed by analysis of the permeability as described (15). Results are expressed as mean ± S.E. and all experiments were repeated at least three times.

The same assay was employed to evaluate the effect of thrombin (2 nM for 3 h) or TRAP (0–1 mM for 3 h) on permeability of endothelial cells pretreated with near physiological concentrations of vitamin K-dependent coagulation protease zymogens (protein C, prothrombin, FVII, FIX, and FX) as well as FX-S195A and PC-S195A (50 nM) for 15 min. The permeability assays were also carried out in the presence of either PTX (100 ng/ml, 16 h) or siRNA for S1P1, EPCR, PAR-1, and PAR-2 (0.2 μg/ml, 30 min) as described (15, 29). EA.hy926 cell permeability in response to TNF-α was carried out using the same methods except that instead of 5 nM thrombin for 10 min, TNF-α (10 ng/ml for 18 h) was used to induce permeability. This permeability assay was also used to analyze the effect of FVIIa (10 ps) and the zymogen FX (175 nM) on EA.hy926 cells transiently transfected with the dc-TF construct. This assay was also used to monitor the signaling effect of thrombin in response to TNF-α on cells pretreated with GDFX, E2FX, E2FX-RK/AA, and GDPC-FX6aa derivatives (50 nM each for 30 min).
Analysis of Expression of Cell Adhesion Molecules—The expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin on EA.hy926 was determined by a whole cell ELISA as described (29, 30). Briefly, confluent monolayers were treated for 4 h with 20 nM APC, thrombin ± PC-S195A, thrombin ± FX-S195A, or TRAP (100 μM) ± FX-S195A (50–175 nM) followed by stimulation with TNF-α (10 ng/ml for 4 h). Following removal of the medium and washing of cells with PBS, they were fixed by adding 50 μl of 1% paraformaldehyde for 15 min at room temperature. Mouse anti-human monoclonal antibodies to VCAM-1, ICAM-1, and E-selectin (Chemicon, Temecula, CA) and peroxidase-conjugated anti-mouse IgG antibody (Sigma) were used to measure the expression of cell adhesion molecules as described (29, 30). All measurements were performed in triplicate wells.

Adhesion Assay—Freshly isolated neutrophil adherence to EA.hy926 cells was evaluated by fluorescent labeling of neutrophils as described (29, 30). Briefly, freshly isolated peripheral blood neutrophils were labeled with 5 μM Vybrant DiD (Molecular Probes) for 20 min at 37 °C in phenol red-free RPMI containing 5% fetal bovine serum. Following twice washing of neutrophils (1.5 × 10⁶/ml, 200 μl/well), they were resuspended in the adhesion medium (RPMI containing 2% fetal bovine serum and 20 mM HEPES) and added to confluent monolayers of EA.hy926 cells in 96-well plates, which were treated for 4 h with 20 nM of either APC or thrombin ± FX-S195A zymogen (175 nM) followed by TNF-α (10 ng/ml for 4 h). In blocking experiments, the monolayers were preincubated for 30 min at 37 °C with appropriate function-blocking antibodies. The fluorescence of labeled cells was measured (total signal) using a fluorescence microplate reader (Molecular Device). After incubation for 60 min at 37 °C, the non-adherent cells were removed by washing four times with pre-warmed RPMI and fluorescent signals of adherent cells were measured by the same methods. The percentage of adherent leukocytes was calculated by the formula: % adherence = (adherent signal/total signal) × 100, as described (31). All measurements were performed in triplicate wells. The same procedures were employed to monitor the binding of the monocytic THP-1 cells to TNF-α-stimulated endothelial cells.

Measuring RhoA and Rac1 Activation—EA.hy926 cells were grown to confluence in 6-well culture dishes and starved overnight in the serum-free medium. The NF-κB pathway activation in the conditioned cell lysates (treated with different PAR-1 agonists and activated by TNF-α as described under figure legends) was monitored by Western blotting employing two antibodies that are specific for either NF-κB p65 (as an index of total cellular NF-κB p65) or its phosphorylated form (as an index of the NF-κB pathway activation) as described (16).

Immunoprecipitation, SDS-PAGE, and Western Blotting—Total cellular proteins were extracted by sonication with PBS containing 25 μM proteasome inhibitor MG132 (Sigma) and complete protease inhibitor mixture (Roche Applied Science) as described (15, 16). Lysates were combined with 3 μg of each specific antibody (H-111 for PAR-1, SAM-11 for PAR-2, EPR11-A for effective cell protease receptor 1, RCR-252 for EPCR, and anti-caveolin-1) and incubated for 2 h at 4 °C. Immunoprecipitates were collected with protein A/G-agarose (Santa Cruz, CA), fractionated on a 10% SDS-PAGE, transferred to membranes, and subjected to Western blotting with appropriate primary and horseradish peroxidase-conjugated secondary antibodies as described (16). The immunoreactive protein bands were visualized by SuperSignal West Pico (Pierce, Rockford, IL).

RESULTS

FX Switches the PAR-1- and PAR-2-dependent Signaling Specificity in Endothelial Cells—We previously demonstrated that the occupancy of EPCR by protein C switches the PAR-1-dependent signal specificity of thrombin from a permeability enhancing to a barrier-protective effect in endothelial cells (15, 29). To determine whether the interaction of other vitamin K-dependent coagulation protease zymogens influences the PAR-1- and PAR-2-dependent signaling specificity of coagulation proteases, we pre-treated EA.hy926 cells with near physiological concentrations of protein C, prothrombin, factors VII, IX, and X before activating the receptors with TRAP highly specific for either PAR-1 (TFLLRN) or PAR-2 (SLIGKV) (32). We discovered that in addition to changing the signaling specificity of PAR-1, protein C also changed the PAR-2 signaling specificity in an endothelial cell hyperpermeability assay (Fig. 1A). Unexpectedly, we discovered that FX also exhibited a similar property. Thus both PAR-1 and PAR-2 activation elicited barrier-protective effects when endothelial cells were treated with either protein C or FX (Fig. 1A). Further studies with catalytically inactive FX-S195 mutant zymogen supported a protective role for FX in endothelial cells. As shown in Fig. 1, B and C, both TFLLRN and SLIGKV enhanced the permeability of endothelial cells in a concentration-dependent manner with maximal effects reaching saturation at ~50 μM TRAP. How-
ever, FX-S195A switched the signaling specificity of both PAR agonist peptides so that neither agonist could elicit a barrier-disruptive effect. These results suggest that, similar to the protective EPCR-protein C system, the activation of both PAR-1 and PAR-2 initiate protective responses if endothelial cells were pretreated with the zymogen FX. Because FXa is known to activate PAR-2, these results indicated that FXa should also exhibit a barrier-protective activity in this assay. Consistent with this proposition, FXa elicited a potent barrier-protective activity in this assay. Consistent with this proposition, FXa elicited a potent barrier-protective activity in this assay. Consistent with this proposition, FXa elicited a potent barrier-protective activity in this assay. Consistent with this proposition, FXa elicited a potent barrier-protective activity in this assay. Consistent with this proposition, FXa elicited a potent barrier-protective activity in this assay.

In addition to signaling through PAR-2, the results presented above with the PAR-1 agonist peptide predict that the zymogen FX can also elicit a protective signaling response indirectly through switching the PAR-1-dependent signaling specificity of thrombin, as has been observed for protein C (15). Indeed, thrombin through the activation of PAR-1 elicited a barrier-protective response on endothelial cells pretreated with FX and/or FX-S195A (Fig. 2A, shown for FX only). Similar to the protein C-mediated protective effect, the protective effect of FX was sensitive to PTX (Fig. 2A). It is known that among G-proteins, only the signaling function of $G_{i/o}$ is sensitive to PTX (33). Thus, these results suggest that the signaling response of FX may be mediated through coupling of PAR-1 to a $G_{i/o}$ protein. Similar to the protective effect of protein C, a receptor cross-talk with S1P is required for the protective effect of FX through PAR-1 because siRNA for S1P1 inhibited this effect (Fig. 2A). Interestingly, whereas the protective effect of the protease, FXa, through the activation of PAR-2 was also sensitive to PTX, nevertheless, siRNA for S1P1 did not inhibit the PAR-2-dependent protective signaling effect of FXa, suggesting that a receptor cross-talk with S1P1 is not required for the protective signaling mechanism observed with...
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FXa (20 nM) inhibited thrombin-mediated up-regulation of all three molecules through the activation of PAR-2 as evidenced by the anti-PAR-2, but not anti-PAR-1, antibody blocking the protective effect of FXa (data not shown). Further studies were initiated to determine whether the inhibition of the expression of these adhesion molecules by thrombin in the presence of FX-S195A correlates with the inhibition of binding of neutrophils to TNF-α-stimulated EA.hy926 cells. The results presented in Fig. 3B demonstrate that, similar to APC, thrombin potently blocks the adhesion of neutrophils to TNF-α-stimulated endothelial cells by a PAR-1-dependent pathway if the cell monolayer was pretreated with FX-S195A prior to incubation with thrombin. A similar approach was employed to monitor the adhesion of the monocytic THP-1 cell line to TNF-α-stimulated EA.hy926 cells, which were pretreated with the FX-S195A zymogen. Similar to results obtained with neutrophils, thrombin inhibited adhesion of THP-1 cells to TNF-α-stimulated endothelial cells pretreated with FX-S195A (Fig. 3C). The function-blocking anti-PAR-1, but not anti-PAR-2 antibody abrogated the anti-inflammatory effect of TNF-α in FX-S195A-pretreated endothelial cells (Fig. 3, B and C). These results clearly suggest that cleavage of PAR-1 by thrombin elicits anti-inflammatory responses in endothelial cells if the cell monolayer is pretreated with the FX zymogen. It should be noted that FXa also inhibited the adhesion of leukocytes to activated endothelial cells (Fig. 3, B and C), in this case, however, the effect was mediated through PAR-2 because only anti-PAR-2 antibody inhibited this effect (data not presented). As expected, MβCD abrogated the PAR-1-dependent protective activity of thrombin on the FX-S195A-pretreated cells, suggesting that the interactions take place within the lipid rafts of endothelial cells (Fig. 3, B and C).

The Barrier-protective Effect of FX/FXa Is Mediated through Modulation of the Rho Family of GTPases—It has been previously demonstrated that the barrier-disruptive effect of thrombin and the opposite barrier-protective effect of APC are mediated through these proteases differentially modulating the cytoskeletal proteins, actin and myosin, via the activation of the Rho family of small GTPases (16, 34). Thus, the proinflammatory molecules have been shown to increase the levels of the phosphorylated myosin light chain through the activation of RhoA, thereby inducing the formation of actin stress fibers that can lead to cellular contraction and disruption of the endothelial interaction of neutrophils with endothelial cells by inhibiting the expression of cell surface adhesion molecules VCAM-1, ICAM-1, and E-selectin (15, 16, 29, 30). The results presented in Fig. 3A indicate that, similar to protein C-S195A, pre-treating endothelial cells with FX-S195A switched the signaling specificity of thrombin, thus conferring anti-inflammatory activities for the protease through the activation of PAR-1. This was supported by the observation that TRAP (TFLLRN) also elicited a protective response if cells were pretreated with either PC-S195A or FX-S195A (Fig. 3A). The concentration dependence of the protective effect of thrombin on S195A pretreated cells revealed that a thrombin concentration of 10–20 nM is required for the maximal inhibition of expression of all three cell adhesion molecules and that increasing the concentration of thrombin to 50 nM eliminated the protective effect of the protease (data not shown). This is consistent with our previous results (16) and suggests that higher concentrations of thrombin may also signal through other cell surface receptor(s) that can counteract its protective effect. Consistent with a PAR-1-dependent response for both protein C and FX mutant zymogens, the function-blocking anti-PAR-1 antibody inhibited the protective effect of thrombin (Fig. 3A). A similar down-regulation of cell adhesion molecules was observed if FXa was directly used to monitor the expression of adhesion molecules. Thus,
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FIGURE 3. TNF-α-mediated up-regulation of cell adhesion molecules on endothelial cells. A, the PAR-1-dependent effect of APC (20 nM, 4 h), thrombin (20 nM, 4 h), and TFLLRN (100 μM, 4 h) on the modulation of the cell adhesion molecules VCAM-1, ICAM-1, and E-selectin in response to TNF-α (10 ng/ml, 4 h) was monitored by ELISA after treating cells with either PC-S195A (50 nM, 30 min) or FX-S195A (175 nM, 30 min) in the absence and presence of the function-blocking anti-PAR-1 antibody (25 μg/ml). B, endothelial cells were activated with TNF-α (10 ng/ml, 4 h) after treating cells with APC (20 nM, 4 h), FXa (20 nM, 4 h), and thrombin (20 nM, 4 h) ± FX-S195A (175 nM, 30 min), and the adherence of neutrophils (panel A) or THP-1 cells (panel C) was monitored as described under "Experimental Procedures.” In the presence of MβCD (10 mM, 1 h) and function-blocking anti-PAR-1 anti-PAR-2 antibodies (25 μg/ml, 30 min), cells were first incubated with these reagents. All results are means ± S.D. of three different experiments.

FIGURE 4. FX recruits PAR-1 to a protective pathway by mediating the dissociation of EPCR from caveolin-1 in lipid rafts of endothelial cells. A, the protective activity of FX-S195A was associated with inhibition of the NF-κB pathway in TNF-α-stimulated endothelial cells (Fig. 4A, bottom two rows). Further studies indicated that FXa, whether added directly to the TNF-α-stimulated endothelial cells or generated by the picomolar concentration of FVIIa on TF bearing cells, also elicited an anti-inflammatory response (Fig. 4B, lanes 3 and 6). In this case, however, the response was mediated through activation of PAR-2 because the anti-PAR-2 antibody inhibited the effect (Fig. 4B, lane 7). Consistent with the PAR-2 specificity of the response, the PAR-2-specific TRAP also activated Rac1 in the presence of FX-S195A, however, in the absence of the zymogen mutant, activation of PAR-2 by the PAR-2 agonist peptide resulted in activation of RhoA (Fig. 4B, lanes 8 and 9). Similar to results in Fig. 4A, the protective activity of FXa is mediated through inhibition of the NF-κB pathway (Fig. 4B). These results clearly suggest that FX/Xa indirectly through PAR-1 and directly through the activation of PAR-2 elicits anti-inflammatory responses in stimulated endothelial cells through modulation of the Rho and NF-κB pathways.

TX Recruits PAR-1 to a Protective Pathway by Mediating the Dissociation of EPCR from Caveolin-1 in Lipid Rafts of Endothelial Cells—To understand the mechanism by which FX recruits PAR-1 to a protective signaling pathway, we immunoprecipitated the total protein extract of non-treated and FX-S195A-treated endothelial cells with anti-caveolin-1, anti-PAR-1, anti-EPCR, and anti-PAR-2 antibodies and subjected them to a 10% reducing SDS-PAGE. Western blot analyses of immunoprecipitates indicated that with the exception of PAR-2, the antibody specific for each receptor co-immunoprecipitates the other two receptors from the extract derived from non-treated endothelial cells (Fig. 5, A–C), suggesting that both EPCR and PAR-1 are associated with caveolin-1 in the same membrane microdomains. Interestingly, when endothelial cells were pretreated with FX-S195A, whereas both EPCR and PAR-1 pairs and caveolin-1 and PAR-1 pairs remained associated (Fig. 5A), EPCR
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A

B

C

D

E

and caveolin-1 were not co-immunoprecipitated by the same antibody pairs (Fig. 5, B and C), suggesting that the occupancy of an endothelial receptor by the FX zymogen leads to dissociation and/or migration of EPCR out of the caveolar compartment, a process that appears to couple PAR-1 to the G<sub>i</sub> protein, thereby changing the specificity of the PAR-1 signaling from a proinflammatory to an anti-inflammatory response. In contrast to PAR-1, the same experimental approach did not provide any evidence of PAR-2 associating with any one of the other three receptors on either FX-S195A-treated or non-treated cells (Fig. 5, A–D).

FX-mediated Recruitment of PAR-1 to a Protective Pathway Is Independent of Gla Domain—We previously demonstrated that interaction of the Gla domain of protein C with EPCR is required for its ability to recruit PAR-1 to a protective pathway on endothelial cells (15, 16). To determine whether this is also true for FX/Xa, we compared the activities of recombinant FX zymogen derivatives lacking either Gla domain (GDFX) or both Gla and EGF1 domains (E2FX) in the hyperpermeability assay. Interestingly, both deletion derivatives of FX switched the PAR-1-dependent signaling specificity of thrombin from a permeability enhancing effect to a barrier-protective effect, suggesting that interaction of the Gla domain of FX with endothelial cells is not required for recruiting PAR-1 to a protective pathway (Fig. 6A). It has been previously reported that the 6 residues of the inter-EGF sequence Leu-83 to Leu-88 (Leu-Phe-Thr-Arg-Lys-Leu) in FXα competes with binding of the radiolabeled FXα to an endothelial cell surface (36). To determine whether interaction of the Gla-domainless FX derivatives with the same receptor accounts for its signaling activity on endothelial cells, the same experiments were conducted with a Gla-domainless protein C in which the 6 residues of the inter-EGF sequence of protein C were replaced with the corresponding residues of FX (GDPC-FX<sub>66α</sub>). This Gla-domainless protein C mutant recruited PAR-1 to a protective pathway similar to GDFX and E2FX (Fig. 6A), suggesting that interaction of this sequence with an endothelial cell surface receptor is required for receptor mobilization by FX, and its protective signaling effect. Further studies with an E2FX mutant having Ala substitutions for the two N terminus residues, Arg-86 and Lys-87 (E2FX-RK/AA), suggested that this mutant is inactive in the permeability assay (Fig. 6A), suggesting that the two basic inter-EGF residues interact with the putative FX receptor on endothelial cells. Consistent with results presented above, both wild-type and Gla-domainless FXα derivatives exhibited a direct PAR-2-dependent protective effect on endothelial cells as evidenced by the blocking anti-PAR-2, but not anti-PAR-1 antibody reversing the protective effect of FXα derivatives (Fig. 6B). The identity of the FX/FXα receptor on endothelial cells is not known. However, several years ago, a receptor, termed effector cell protease receptor-1 (EPR-1), was reported to be the putative FXα receptor responsible for the cellular effect of the protease in endothelial cells (37). Although further studies did not confirm the identity of this receptor (38), nevertheless, our results showed that a commercially available antibody (EPR11-A...
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![Graph: Permeability (OD600 nm) vs Time]

FIGURE 6. Thrombin reverses the hyperpermeability effect of TNF-α in endothelial cells pretreated with FX derivatives. A, EA.hy926 cells were incubated with FX derivatives (50 nM each for 30 min) followed by incubation with thrombin (2 nM, 3 h) and activation of cells by TNF-α (10 ng/ml for 18 h). The permeability was monitored as described under “Experimental Procedures.” B, the same as A except that cells were incubated with FXa derivatives (20 nM) with or without preincubating cells with blocking anti-PAR-1, anti-PAR-2, and anti-EPR-1 (EPR11-A) antibodies. C, co-immunoprecipitation and Western blotting of total cellular proteins derived from transformed (EA.hy926) and primary HUVECs using anti-PAR-2 and anti-EPR-1 antibodies. IB, immunoblot. All results in A and B are means ± S.D. of three different experiments.

Obtained from Alpha Diagnostica Int. Inc., San Antonio, TX) inhibited the protective effect of FXa derivatives (Fig. 6B). Interestingly, further studies indicated that both PAR-2 and the putative FX/FXa receptor may be associated with each other because either anti-PAR-2 or anti-EPR-1 antibody co-immunoprecipitated both receptors derived from cell lysates of either transformed or primary HUVECs (Fig. 6C).

DISCUSSION

We have demonstrated in this study that interaction of the zymogen FX with an endothelial cell surface receptor can recruit both PAR-1 and PAR-2 to protective signaling pathways as evidenced by the specific agonist peptides to both receptors reversing the permeability enhancing effect of thrombin to a barrier-protective effect in cells pretreated with FX or FX-S195A (Fig. 1A). This property of FX is reminiscent of the signaling mechanism of protein C/APC, which also recruits PAR-1 to a cytoprotective pathway upon interaction with EPCR on endothelial cells (15, 16). The results presented in Fig. 1 further indicate that protein C also changes the specificity of the PAR-2 signaling. Thus activation of both PAR-1 and PAR-2 by coagulation proteases or by the specific receptor agonist peptides elicited only protective cellular responses as long as the specific receptor for either protein C or FX was occupied by its respective ligand. We previously demonstrated that the protective activity of protein C is mediated through the Gla domain-dependent interaction of the protein C/APC with EPCR (15, 16), an interaction that leads to dissociation of EPCR from caveolin-1 in lipid rafts of endothelial cells, thereby coupling PAR-1 to the PAR-2 effects suggest that a cross-talk between PAR-1 and 1P). But not between PAR-2 and 1P, is required for modulation of the G-protein coupling specificity of these receptors.

It has been previously demonstrated that FXa, via a loop connecting the EGF1 to EGF2 domain, interacts with a receptor on endothelial cells to elicit intracellular signaling responses (21, 36). The identity of this receptor is not known and the hypothesis that EPR-1 is the putative FXa receptor responsible for the cellular effect of the protease in endothelial cells (37) could not be confirmed by the failure to detect transcripts that would correspond to the sequence of EPR-1 in endothelial cells (38). Nevertheless, our mutagenesis studies with both E2FX and GDFX and the GDPC chimera, possessing the 6 inter-connecting EGF residues of FX, support the hypothesis that interaction of this sequence (in particular two basic Arg and Lys residues) with an endothelial cell surface receptor is required for the protective signaling effect of FX (Fig. 6). Further support for the existence of a FX/FXa receptor associated with PAR-2 on endothelial cells is provided by the observation that a commercially available antibody (EPR11-A) blocked the protective effect of FXa and co-immunoprecipitated PAR-2 from endothelial cell lysate (Fig. 6, B and C). Thus, similar to the protein C occupancy of EPCR, the FX/FXa occupancy of this putative endothelial cell surface receptor alters the G-protein coupling specificities of both PAR-1 and PAR-2 so that the activation of both receptors by either thrombin (PAR-1) or FXa (PAR-2) elicits protective responses in the cytokine-activated endothelial cells. The observations that FXa, whether added directly to
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![Diagram of PAR-1 and PAR-2 activation by coagulation proteases](image)

**FIGURE 7.** A hypothetical model of PAR-1 and PAR-2 activation by coagulation proteases when either EPCR or an unknown FX/FXa receptor is occupied by natural ligands. In the absence of protein C/PC or FX/FXa, EPCR associates with caveolin-1 (Cav-1) within the lipid rafts of endothelial cells (left panel in the box). In this case, thrombin appears to signal via Gαi and/or G12/13, thereby enhancing cellular permeability through the activation of RhoA and eliciting proinflammatory responses through the activation of NF-κB (16). The occupancy of either EPCR by protein C (PC) or FX receptor by its ligand results in dissociation of EPCR from caveolin-1 (right panel). This process appears to be linked with the coupling of PAR-1 to Gαi. In this case, the activation of PAR-1 by thrombin increases the barrier integrity of endothelial cells by activating Rac1 GTPase and eliciting an anti-inflammatory response through the inhibition of NF-κB (16). A similar protective effect is mediated when FX binds to its receptor on endothelial cells. In this case, not only the activation of PAR-1 by thrombin is protective, but FXa also elicits a protective response directly through the activation of PAR-2, thereby activating Rac1 GTPase and inhibiting the NF-κB pathway. See text for more details.

activated endothelial cells or generated from the FX zymogen activation by FVIIa on endothelial cells bearing TF, elicited a barrier-protective effect in the hyperpermeability assay, inhibited the expression of cell adhesion molecules, and impeded the interaction of leukocytes with the TNF-α-activated endothelial cells, are consistent with this hypothesis. Similar to the protective activity of the APC/protein C system, both the indirect PAR-1-dependent and direct PAR-2-dependent protective activities of FX/FXa (Fig. 4) were mediated through the activation of Rac1 and inhibition of the activation of RhoA and NF-κB pathways as evidenced by the agonist peptides to both PAR-1 and PAR-2 specifically mediating the activation of RhoA in non-treated endothelial cells, but the same peptides preferentially activating Rac1 in the FX-S195A-treated endothelial cells. A hypothetical model summarizing these results is presented in Fig. 7.

The results presented above underscore the potential problems associated with in vitro studies using different cellular models to draw physiologically relevant conclusions related to the PAR-dependent signaling specificity of coagulation proteases. It is not therefore surprising that different studies using different cell lines have often reported inconsistent results (both cytoprotective and cytotoxic) for the activation of either PAR-1 or PAR-2 by coagulation proteases under different conditions. Given the importance of coagulation proteases in maintaining hemostasis, and the requirement for their rapid activation during injury and inflammation, it is likely that the co-receptor signaling system described above on endothelial cells is a safeguard mechanism to ensure that PAR-1 and PAR-2 activation by coagulation proteases would only elicit protective responses on the healthy vasculature bearing the appropriate cell surface receptors. Thus, we hypothesize that the activation of both PAR-1 and PAR-2 by coagulation proteases, under physiological conditions where the cell surface receptors (i.e. EPCR and an FX receptor) are occupied by their natural ligands, will elicit protective signaling responses by the inhibition of the NF-κB and RhoA pathways and the activation of the Rac1 pathway. In further support of this hypothesis, we recently demonstrated that occupancy of EPCR by protein C also inhibits thrombin mobilization of P-selectin from Weibel-Palade bodies in endothelial cells, thus inhibiting the interaction of leukocytes with the endothelium (39). Whether the FX receptor occupancy on endothelial cells also inhibits thrombin mobilization of P-selectin from Weibel-Palade bodies needs further investigation.

Acknowledgment—We thank Audrey Rezaie for proofreading the manuscript.

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