PAR1 Cleavage and Signaling in Response to Activated Protein C and Thrombin*

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Activated protein C (APC), a natural anticoagulant protease, can trigger cellular responses via protease-activated receptor-1 (PAR1), a G protein-coupled receptor for thrombin. Whether this phenomenon contributes to the physiological effects of APC is unknown. Toward answering this question, we compared the kinetics of PAR1 cleavage on endothelial cells by APC versus thrombin. APC did cleave PAR1 on the endothelial surface, and antibodies to the endothelial protein C receptor inhibited such cleavage. Importantly, however, APC was 104-fold less potent than thrombin in this setting. APC and thrombin both triggered PAR1-mediated responses in endothelial cells including expression of anti-apoptotic (tumor necrosis factor-α-induced a20 and iap-1) and chemokine (interleukin-8 (IL-8) and cxcl3) genes, but again, APC was 104-fold less potent than thrombin. The addition of zymogen protein C to endothelial cultures did not alter the rate of PAR1 cleavage at low or high concentrations of thrombin, and PAR1 cleavage was substantial at thrombin concentrations too low to trigger detectable conversion of protein C to APC. Thus, locally generated APC did not contribute to PAR1 cleavage beyond that effected by thrombin in this system. Although consistent with reports that sufficiently high concentrations of APC can cleave and activate PAR1 in culture, our data suggest that a significant physiological role for PAR1 activation by APC is unlikely.

Thrombin and activated protein C (APC) are serine proteases with key roles in hemostasis and thrombosis (1). Thrombin serves as the central effector of the coagulation cascade by converting fibrinogen to fibrin and by activating platelets and other cells through protease-activated receptors (PARs) (2, 3). PAR1, the prototype for this receptor family, is a heptahelial G protein-coupled receptor for thrombin (4). Activation of PAR1 is accomplished when thrombin binds to and cleaves the N-terminal exodomain of PAR1 at a specific site. This cleavage generates a new receptor N terminus and thereby unmasks a “tethered ligand” that binds to the heptahelial segment of the receptor to effect transmembrane signaling and G protein activation. Importantly, cleavage of the activation site of PAR1 is both necessary and sufficient for receptor activation, and several proteases with trypsin-like specificity are capable of cleaving this site and activating PAR1 in vitro (5–7).

APC provides critical negative feedback regulation of thrombin generation. Thrombin converts zymogen protein C to APC, which in turn cleaves and inactivates coagulation factors Va and VIIIa, cofactors necessary for thrombin production (1). Generation of APC is localized to the surface of endothelial cells by the action of two integral membrane cofactors, thrombomodulin (TM) and endothelial protein C receptor (EPCR), which bind thrombin and protein C, respectively (8, 9).

Interest in the natural anticoagulant and other biological activities of APC has intensified due to the recent finding that APC administration can reduce mortality in sepsis (10). Subsequently, it was reported that APC is capable of activating PAR1 on endothelial cells, and it was postulated that this phenomenon might contribute to the protective effect of APC in sepsis (11, 12). Many PAR1-triggered responses in endothelial cells, such as production of platelet-activating factor and chemokines and display of adhesion molecules for leukocytes and platelets, would be predicted to promote inflammation, thrombosis, and tissue damage (13–15). However, PAR1 does induce inhibitors of apoptosis such as A20 and IAP-1 (16); these and related responses might be important for protecting endothelial cells in an environment rich in proapoptotic cytokines and/or ischemia, as might occur in sepsis and at sites of tissue damage (11, 16–18). Of note, thrombin is a potent activator of PAR1 on endothelial cells (4, 19–21), and generation of APC requires the presence of thrombin at the endothelial surface (1). Might APC contribute to PAR1 activation beyond that already effected by thrombin? Might APC versus thrombin activation of PAR1 somehow favor protective versus proinflammatory responses in endothelial cells? Toward addressing these questions, we have compared the kinetics and the effects of endothelial PAR1 activation by thrombin versus APC. Our results militate against an important role for APC in PAR1 signaling.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from Sigma unless otherwise noted. Human thrombin was from Enzyme Research Laboratories

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1 The abbreviations used are: APC, activated protein C; PAR, protease-activated receptor; AP, alkaline phosphatase; TM, thrombomodulin; EPCR, endothelial protein C receptor; A20, tumor necrosis factor-α-induced A20; IAP-1, inhibitor of apoptosis protein-1; HUVEC, human umbilical vein endothelial cell; IL-8, interleukin 8; BSA, bovine serum albumin; DME, Dulbecco’s modified Eagle’s.

2 Y. Srinivasan and S. R. Coughlin, unpublished data.
(South Bend, IN). APC was obtained from Enzyme Research Laboratories or prepared from plasma-derived protein C as described (22). The activities of APC from the two sources were indistinguishable in the presence of hirudin (see below). Zymogen protein C was from Hematologic Technologies Inc. (Essex Junction, VT). The chromogenic APC substrate, Spectrozyme PCs (H-β-Lys (γ-Chloro) pro-Arg-pNA.2AcOH), was from American Diagnostica (Stamford, CT). Recombinant hirudin was from American Diagnostica or Calbiochem. SFLLRN peptide was synthesized as a C-terminal amide and purified by high pressure liquid chromatography (ANASPEC, San Jose, CA). Cells, cell culture media, and accompanying supplements were from the University of California, San Francisco Cell Culture Facility unless otherwise specified.

Quantitation of Soluble Alkaline Phosphatase Released by PAR1 Cleavage—The amount of alkaline phosphatase liberated by cleavage of AP-PAR1 was determined as described (23). Briefly, conditioned medium was collected after incubation of AP-PAR1-transfected HUVECs with APC or thrombin and centrifuged to remove any cell debris. Supernatant was collected, and alkaline phosphatase activity was measured using the chemoluminescent substrate, diosmine 3-4-methoxy-1-(2,3-dioxetane-2,5′-chloro) tricyclo(3.3.1.13,7-decan)-4-yl phenyl phosphate. Subpicogram quantities of recombinant AP could be detected by this assay, and the relationship between chemiluminescence and protein was linear from 0.1 pg to at least 10 ng. Activity levels in the experiments presented here generally ranged from 1 to 30 pg. Background luminescence in medium conditioned without transfected HUVECs was equivalent to less than 1 pg of alkaline phosphatase.

Measurement of APC Activity—In experiments designed to assess protein C conversion to APC in culture, APC activity in conditioned medium was measured as described (9). Briefly, 135 μl of cleared conditioned HEPES-buffered saline solution medium was mixed with 15 μl of 40 units/ml hirudin (4 units/ml final concentration) to block any endogenous activity that might be contributed by thrombin. 135 μl of this sample was in turn added to 15 μl of a solution containing the chromogenic APC substrate, Spectrozyme PCs, to give a final substrate concentration of 1 mM. The rate of change in absorbance with time (ΔOD at 405 nm/min) at room temperature was measured using a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). The amount of APC present in samples was derived from a standard curve generated with 1–1000 units/ml APC, which was linear up to ~900 units/ml. Detectable activation of protein C in this system was only observed when both thrombin and cells were present during the incubations.

Northern Blotting—Northern probes for IL-8, CXCL3, IAP-1, and A20 were synthesized as a C-terminal amide and purified by high pressure liquid chromatography (Stratagene, La Jolla, CA). HUVECs were plated on gelatin-coated 60-mm dishes, incubated in serum-free endothelial basal medium (Clonetics) (plus 0.1% BSA) for 14 h, and then further incubated with APC or thrombin for 120 min (IL-8 and CXCL3) or 90 min (IAP, A20); preliminary studies had shown that the maximum induction of each transcript occurred at these times. From each 60-mm dish, 10 μg of total RNA was then isolated using TriZol (Life Technologies) and separated by 1% agarose gel electrophoresis and transferred to Hybond-NX Membrane (Amersham Biosciences). Membranes were hybridized in Perfect-Hybrid solution (Sigma) with radiolabeled probes for 3 h at 68 °C, washed five times at room temperature (1× SSC, 0.1% SDS, 10 min) and twice at 50 °C (0.1× SSC, 0.1% SDS, 15 min), and analyzed with a Storm PhosphorImager (Amersham Biosciences).

RESULTS

To directly compare the ability of APC and thrombin to cleave PAR1 on endothelial cells, we utilized a PAR1 cleavage reporter expressed in these cells. This substrate, AP-PAR1, is a chimeric protein in which the C terminus of secreted alkaline phosphatase (absent its transmembrane domain) is joined to the N-terminal exodomain of PAR1 (absent its signal peptide). The junction between alkaline phosphatase and PAR1 is N-terminal to the thrombin cleavage site of PAR1 (23). Thus, alkaline phosphatase is tethered to the cell membrane by the N-terminal exodomain of PAR1 and released when this domain is cleaved by thrombin or other proteases, and soluble alkaline phosphatase activity in cell-conditioned medium provides an easily measured index of PAR1 cleavage (23, 25).

The range of thrombin concentrations explored in our AP-PAR1 cleavage studies was 10 pm to 30 nm. The level of pro-thrombin in the circulation is 1–2 μM, and concentrations of
active thrombin in the 10 nM range are almost certainly physiologically relevant (26). The range of APC concentrations explored was 1–100 nM. The level of zymogen protein C in the circulation is ∼65–80 nM, and circulating APC levels are generally less than 0.2 nM even in sepsis (27).

Incubation of AP-PAR1-expressing HUVECs for 1 h with 1 or 10 nM APC yielded little PAR1 cleavage, but at 100 nM APC, ∼20% of maximal PAR1 cleavage, defined as that caused by 10 nM thrombin, was detected (Fig. 1A). 0.01 nM thrombin yielded a similar amount of PAR1 cleavage under the same conditions, and ∼50% maximal PAR1 cleavage occurred at 0.1 nM thrombin. Thus, thrombin was ∼104-fold more potent than APC in this assay. In a second set of experiments, 30 nM thrombin yielded virtually complete cleavage of cell surface PAR1 within 6 min, 75 pM thrombin yielded ∼60% of maximal cleavage at 60 min, and 100 nM APC yielded only ∼30% maximal cleavage at 60 min (Fig. 1B). Again thrombin appeared to be 103-104-fold more potent than APC.

The relatively low activity of APC versus thrombin raised the question of whether a small amount of thrombin contaminating APC preparations might contribute to the activity of APC seen in these studies. This is unlikely. In preliminary experiments, we had indeed found hirudin-inhibitable activity in some APC preparations, but in these cases, increasing concentrations of hirudin did not block all PAR1-cleaving activity. In all of the experiments shown, APC was preincubated with a concentration of hirudin sufficient to completely block 10 nM thrombin (this concentration of hirudin had no effect on APC as assessed by amidolytic activity (see “Experimental Procedures”). In addition, the ability of APC to cleave endothelial PAR1 was inhibited by an antibody (JRK1494) that blocks protein C/APC binding to the EPCR but not by a non-blocking EPCR antibody (JRK1500); JRK1494 did not inhibit PAR1 cleavage by thrombin (Fig. 2 and data not shown). This finding is consistent with the observations of Riewald et al. (11) with regard to APC-triggered extracellular signal-regulated kinase (ERK) activation in endothelial cells. Thus, the low level of PAR1 cleaving activity in APC preparations is likely due to the actions of APC itself rather than by thrombin or other proteases that might contaminate such preparations.

It was possible that the AP-PAR1 molecule was relatively resistant to APC cleavage when compared with wild-type PAR1. To test this possibility, we explored the APC concentration dependence of signaling in untransfected HUVECs in which signaling in response to thrombin and related proteases depends largely on native PAR1 (20, 33, 34). As is apparent from the data in Figs. 3–5, activation of signaling in such cells required at least 1000-fold more APC than thrombin, in accord with the relative potency of APC and thrombin for PAR1 cleavage. This result strongly suggests that the different potencies of thrombin and APC for cleavage of our AP-PAR1 chimera hold for cleavage and activation of wild-type PAR1. To further probe this issue, we assessed the rate of loss of SPAN12 binding to the surface of HUVECs exposed to thrombin or APC. SPAN12 is a monoclonal antibody to a thrombin-sensitive epitope that spans the activating cleavage site in PAR1 (35). Incubation of cells with 100 nM APC at 37°C caused loss of ∼50% of SPAN12 binding in 30 min as assessed by flow cytometry; only 100 pM thrombin was required to achieve the same or greater effect. 10 nM thrombin reduced SPAN12 binding to background levels (data not shown). These results again suggest that APC is ∼1000-fold less potent than thrombin for cleavage and activation of PAR1 on endothelial cells.

The magnitude of thrombin-triggered cellular responses correlates with the rate of PAR1 cleavage (24). This predicts that APC should be 103-104-fold less potent than thrombin for triggering PAR1-mediated responses in endothelial cells. To test this prediction, we first examined phosphoinositide hydrolysis as a convenient end point of PAR1 signaling (PAR1 is the major effector of thrombin-stimulated phosphoinositide hydrolysis in endothelial cells (21)) (Fig. 3). Increases in phosphoinositide...
hydroylsis were readily detected at thrombin concentrations of 0.1 nM and above. Responses to APC could be detected at 30 nM and above, and 100 nM APC triggered responses approximately equivalent to those elicited by 0.1 nM thrombin. Thus, in this functional assay, APC again appeared to be on the order of 10^5-fold less potent than thrombin.

We next analyzed gene induction by thrombin and APC both as an additional end point for comparing the potency of these proteases for triggering PAR-mediated responses in endothelial cells and to probe for possible qualitative differences between thrombin and APC signaling in these cells. CXCL3 and IL-8, proinflammatory chemokines known to be induced by thrombin in endothelial cells (28), were induced by both thrombin and APC in these cells; the response elicited by 100 nM APC was less than that elicited by 0.1 nM thrombin (Fig. 4). Similar results were obtained when a20 and iap-1, two antiapoptotic genes known to be modulated by APC (16), were examined (Fig. 5). Thus, responses to APC and thrombin were qualitatively similar, but APC was remarkably less potent than thrombin.

Given that APC generation requires the presence of thrombin on the endothelial surface, the relative lack of potency of APC toward PAR1 raises the question of whether APC is likely to contribute to PAR1 activation beyond that already effected by thrombin. In the studies described above, preactivated APC was added to cell culture medium. Several mechanisms might alter the relative potency of locally generated APC versus thrombin for PAR1 activation. It is possible that locally generated APC might be positioned favorably by EPCR and/or TM for PAR1 cleavage on the endothelial surface, and thrombin might be positioned unfavorably for PAR1 cleavage by its interaction with TM (12). Indeed, it has been proposed that at low thrombin levels in vivo, activation of endothelial cell PAR1 might be mediated primarily by thrombin-activated protein C in a coupled system directed by these transmembrane cofactors (12). To determine whether local generation of APC might augment PAR1 cleavage on endothelial cells, we first asked whether physiological concentration (~80 nM) of zymogen protein C might enhance AP-PAR1 cleavage on the endothelial surface at various thrombin concentrations (Fig. 6A). No difference in PAR1 cleavage was observed in the presence or absence of zymogen protein C across a range of thrombin concentrations. To further probe this issue, we used a still higher level of zymogen protein C and, in parallel, measured conversion of protein C to APC (Fig. 6B). Again no difference between PAR1 cleavage in the presence or absence of protein C was detected. Moreover, no significant APC conversion was detected at thrombin concentrations of 0.1 nM and below, and at

![Fig. 3. Phosphoinositide hydrolysis in response to APC versus thrombin.](image)

HUVECs were incubated with APC or thrombin at the indicated concentrations for 1 h, and protease-induced [3H]inositol phosphate accumulation in the presence of lithium chloride was determined. Results (mean ± S.E., n = 12) are presented as fold increase over untreated cultures and represent pooled data from three replicate experiments. Standard errors were small where error bars are not seen. Hir, hirudin.

![Fig. 4. Induction of cxcl3 and il-8 gene expression in HUVECs by APC versus thrombin (aT).](image)

HUVECs were incubated with APC or thrombin (hirudin (Hir.) for 120 min and CXCL3 (top band), and IL-8 (bottom band) mRNA levels were assessed by Northern blot. For quantitation, cxcl3 and il-8 band intensities were normalized to the band intensity for glyceraldehyde-3-phosphate dehydrogenase mRNA, which was similar in all samples (not shown), and results were expressed as fold increase over untreated cells. A, blot from one representative experiment. B and C, results (mean ± S.E.) from quantitation of three replicate experiments.

0.1 nM thrombin, PAR1 cleavage was nearly maximal. Thus, in this model system, there is no evidence that local generation of APC can contribute to PAR1 cleavage beyond that achieved by thrombin alone.

**DISCUSSION**

Direct measurement of kinetics of PAR1 cleavage on “real” endothelial cells revealed APC to be 10^3-10^4-fold less potent than thrombin. A comparison of endothelial responses to APC and thrombin revealed a similar difference in potency. These results are consistent with reports that a sufficiently high concentration of APC can cleave PAR1 and can trigger PAR1-dependent responses in endothelial cells in culture (18, 29–31). They do not speak to the issue of whether APC might regulate the behavior of endothelial (32) or other cells by PAR1-independent mechanisms.

The 10^3-10^4-fold difference in potency between APC and thrombin for PAR1 cleavage on endothelial cells is consistent with earlier studies in which APC was ~5,000 to more than 25,000 times less active than thrombin in solution phase assays of PAR1 peptide cleavage (29, 36–38). Thus, although the cleavage of endothelial PAR1 by APC was EPCR-dependent, it appears that EPCR or other endothelial cofactors do not have a major effect on the inherent enzymatic activity of APC and thrombin toward PAR1.

Importantly, our results raise doubt as to whether PAR1 cleavage by APC is important in vivo, at least without additional cofactors not evident in our system. The concentration of exogenous APC required to trigger PAR1 cleavage and signaling, 10–100 nM, was on the order of the concentration of its
zymogen protein C in plasma (~80 nM) and substantially higher than APC concentrations reported to occur in vivo. In the PROWESS sepsis trial (10), for example, circulating APC levels above 10 ng/ml (~0.2 nM) were rare in the placebo group that did not receive APC infusion. Thus, even in sepsis, the concentration of APC in the circulation is substantially below the concentration required for significant PAR1 activation. In the same model system in which we examined APC activity, 0.1 nM thrombin reliably triggered PAR1 cleavage and endothelial cell responses. This concentration of thrombin is less than one-thousandth the concentration of its zymogen prothrombin in the circulation and well below the concentration of thrombin estimated to be present in clots (26). We were unable to detect evidence that local generation of APC enhances its activity toward PAR1 relative to that of thrombin; indeed, PAR1 cleavage occurred at a concentration of thrombin that did not yield detectable activation of zymogen protein C to APC (Fig. 6). At face value, these data suggest that endogenous APC is unlikely to contribute to PAR1 cleavage beyond that effected by thrombin and militate against a physiological role for PAR1 activation by APC. What about PAR1 as a target for the pharmacological activity of APC? In patients that received APC infusion in the PROWESS trial, the median steady state APC level was 45 ng/ml (~0.9 nM), again well below the concentration of APC required for significant activity at PAR1. This argues against a role for PAR1 cleavage in the pharmacological activities of APC. There are however, a number of caveats.

First, our studies to compare the potency of thrombin and APC as PAR1 agonists were performed in a defined in vitro system in the absence of flow and natural protease inhibitors. This system might overestimate the difference between thrombin and APC in vivo because thrombin activity in the circulation is terminated more rapidly than APC activity (41, 42). Similarly, it is possible that circulating protease inhibitors or flow itself would promote removal of free thrombin from the endothelial surface, whereas relatively sparing TM-bound thrombin in a way that favors thrombin interaction with protein C over interaction with PAR1; this might favor a local coupled system in vivo in a manner not detected in vitro. It is also possible that leukocytes, neurons, or other non-endothelial cell types (see below) are the relevant cellular targets for APC and that different results might be obtained if such cells were examined.

Second, APC-mediated activation of PAR1 is EPCR-dependent, and its effectiveness might depend on EPCR concentration. Although the EPCR expression level in cultured endothelial cells

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**Fig. 5.** Induction of $a_20$ and iap gene expression in HUVECs by APC versus thrombin ($\alpha$T). HUVECs were incubated with APC or thrombin ± hirudin (Hir.) for 90 min, and $a_20$ (A) and iap (B) and glyceraldehyde-3-phosphate dehydrogenase (not shown) mRNA levels were determined as in Fig. 4. A representative Northern blot is shown at top, and quantitated results (mean ± S.E.) from four (A) or three (B) replicate experiments are shown at bottom.

**Fig. 6.** Effect of local conversion of protein C to APC by thrombin on PAR1 cleavage and comparison of protein C versus PAR1 cleavage at various thrombin concentrations. A, HUVECs expressing AP-PAR1 were incubated for 15–20 min with either medium alone or 80 nM protein C and then incubated with thrombin at the indicated concentrations for 1 h. Conditioned medium was collected, and soluble alkaline phosphatase (Alk. Phos.) activity was measured (mean ± S.E.; n = 3). A replicate experiment yielded similar results. B, HUVECs expressing AP-PAR1 were incubated for 15–20 min with HEPES-buffered saline solution buffer with or without 200 nM protein C, after which thrombin was added at the indicated final concentrations for an additional 1 h. Conditioned medium was collected and analyzed for soluble alkaline phosphatase activity (triangles) and APC activity (squares). PAR1 cleavage assay results (y-axis at left) are expressed as a fraction of soluble alkaline phosphatase activity released by 10 nM thrombin in the absence of protein C (mean ± S.E.; n = 6) and represent two replicate experiments. The amount of APC produced in the culture (right y-axis) was determined using a standard curve that was generated with purified APC.
seems a reasonable starting point for modeling EPCR-dependent PAR1 cleavage, EPCR concentration varies widely in vivo, rising as the vessel size increases (39). To address the dependence of PAR1 cleavage by APC on EPCR expression levels, we used control HEK293 cells and an EPCR-overexpressing HEK293 cell line that expressed EPCR at ~7× the density found on HUVECs. Both HEK lines were transiently transfected with the AP-PAR1 construct, and cleavage of this substrate by APC and thrombin was compared. When AP-PAR1 was expressed in control HEK cells, little cleavage was detected even when cells were incubated with 100 nM APC for 60 min. By contrast, substantial cleavage of AP-PAR1 by APC was detected in EPCR-overexpressing HEK cells, but APC was still 100–200-fold less potent than thrombin in this system (when compared with ~1,000 in HUVECs) (data not shown). Thus, PAR1 cleavage by APC could indeed be enhanced by increasing the density of EPCR expression, but whether EPCR is ever expressed at a density high enough to allow APC to function as a physiological activator of PAR1 in vivo is unknown.

Third, differences in thrombin and APC potency in PAR1 cleavage might be influenced by thrombin interactions with other endothelial cell receptors such as TM. Certainly, thrombin binds to TM on endothelium with moderately high affinity (Kd ~0.5 nM). Both TM and PAR1 interact with the anion binding exosite of thrombin; thus, TM-bound thrombin should be relatively poor at recognizing and cleaving PAR1. However, most of the thrombin added to the cultures in these experiments will be free and not associated with the TM (40). Thrombin interaction with TM, therefore, should have little impact on PAR1 cleavage under these conditions. In support of this conclusion, the thrombin concentration dependence of PAR1 activation did not show a threshold thrombin concentration required before PAR1 cleavage was initiated. Moreover, PAR1 cleavage was initiated at ~10 pM thrombin and was substantial by 0.1 nM thrombin, concentrations below those expected to yield substantial binding to TM. Our data examining the thrombin concentration dependence of PAR1 cleavage to that of protein C conversion to APC in endothelial cultures (Fig. 6) are consistent with this view. Thus, endothelial TM is unlikely to affect the relative potency of thrombin and APC in vitro or in vivo.

Fourth, our results suggest that APC is a poor PAR1 agonist that triggers responses in endothelial cells that are qualitatively similar to, but smaller than, those triggered by thrombin. Might PAR1 activation by APC accomplish something distinct from that accomplished by PAR1 activation by thrombin? It is formally possible that low level PAR1 and high level PAR1 activation in endothelial cells might yield qualitatively different outcomes. For example, although both APC and thrombin induce both proinflammatory chemokine and antiapoptotic genes, one could imagine that low level chemokine and antiapoptotic gene induction by APC might be insufficient to promote inflammation but sufficient to protect against apoptosis, whereas high level induction by thrombin might do both.

In vivo studies that address the role of physiological or pharmacological activation of PAR1 by APC are few. It was reported that administration of APC had a protective effect in a mouse model of brain ischemia-reperfusion injury and that this effect was attenuated by PAR1 antibodies and in Par1−/− knockout mice (the protective effect of APC was also reported to be PAR3-dependent in this model, and it was suggested that neurons might be the direct target of the actions of APC) (18, 43). These results support a role for PAR1 in pharmacological effects of APC, at least in this mouse model. The level of APC in the circulation achieved in this model is not known. Interest-ingly, in the same study, PAR1 antibodies did not exacerbate injury in mice that did not receive APC infusion, suggesting that activation of PAR1 by endogenous APC is unimportant in this model. In a similar model, PAR1 deficiency was reported to be protective (44), suggesting that activation of PAR1 by endogenous proteases might promote rather than protect against injury. In endotoxemia models in mice, PAR1 deficiency neither protected against nor exacerbated the effects of endotoxin (45), whereas inhibition of either APC function (46, 47) or EPCR interaction with APC/protein C (48) exacerbates the septic response. Thus, we are unaware of data supporting a physiological role for PAR1 activation by endogenous APC.

In summary, with the caveats noted, our data emphasize that, even when positioned by EPCR on the endothelial surface, APC is a poor agonist for PAR1 relative to thrombin and is unlikely to contribute to PAR1 cleavage beyond that effected by the thrombin that must be present for APC to be generated. The level of APC reported to be achieved pharmacologically in the circulation of septic patients receiving APC infusions (27) is ~20–200-fold below the level of APC required to reliably elicit PAR1 activation. Thus, our data give pause in considering the hypothesis that PAR1 activation mediates physiological or pharmacological effects of APC. In particular, it seems unlikely that PAR1 activation plays a significant role in the beneficial effect of APC infusion in sepsis (11), and alternative mechanisms should be considered (1). For example, damage to endothelial and other cells by activated leukocytes is thought to be an important cause of organ damage in sepsis (49), and APC can interact with protease 3 and Mac1 on the surface of leukocytes (50). From this position, APC may somehow regulate leukocyte function in a manner that is beneficial (51–53).

Note added in Proof—Feistritzer and Riewald recently reported evidence for PAR1-dependent endothelial cell responses to APC concentrations as low as 1 nM, raising the possibility that very low levels of PAR1 activation may have unexpected effects (Feistritzer, C., and Riewald, M. (2005) Blood, in press).

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