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‘Role reversal’ for the receptor PAR1 in sepsis-induced vascular damage

Nicole C Kaneider¹, Andrew J Leger¹, Anika Agarwal¹, Nga Nguyen¹, George Perides², Claudia Derian³, Lidija Covic¹, and Athan Kuliopulos¹

¹Departments of Medicine and Biochemistry, Molecular Oncology Research Institute, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts 02111, USA.

²Department of Surgery, Tufts–New England Medical Center, Boston, Massachusetts 02111, USA.

³Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development, Spring House, Pennsylvania 19477, USA.

Abstract

Sepsis is a deadly disease characterized by considerable derangement of the proinflammatory, anti-inflammatory and coagulation responses. Protease-activated receptor 1 (PAR1), an important regulator of endothelial barrier function and blood coagulation, has been proposed to be involved in the lethal sequelae of sepsis, but it is unknown whether activation of PAR1 is beneficial or harmful. Using a cell-penetrating peptide (pepducin) approach, we provide evidence that PAR1 switched from being a vascular-disruptive receptor to a vascular-protective receptor during the progression of sepsis in mice. Unexpectedly, we found that the protective effects of PAR1 required transactivation of PAR2 signaling pathways. Our results suggest therapeutics that selectively activate PAR1–PAR2 complexes may be beneficial in the treatment of sepsis.

Sepsis remains the leading cause of mortality of patients in intensive care units, causing at least 210,000 deaths annually in the United States¹. Much of the pathology of sepsis has been attributed to a hyper-reaction of the inflammatory system to the invading pathogens, a condition called ‘systemic inflammatory response syndrome’². During the early phases of sepsis, systemic concentrations of inflammatory cytokines and chemokines rapidly increase and the endothelium is activated to cause vascular leakage and septic shock. In late-stage sepsis, the clotting cascade is triggered by the damaged endothelium, leading to disseminated intravascular coagulation (DIC) and multiorgan failure^{3,4}. The vascular damage is caused by many sepsis-related factors, including bacterial endotoxin, tumor

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Correspondence should be addressed to A.K. (athan.kuliopulos@tufts.edu).

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AUTHOR CONTRIBUTIONS

N.C.K. and A.K. conceptualized and designed the experiments; N.C.K., A.J.L., A.A., N.N. and L.C. did the experiments; G.P. and C.D. generated and provided *Par1*^{−/−} and *Par2*^{−/−} mice and intellectual contributions; and N.C.K., L.C. and A.K. analyzed the data and prepared the manuscript.

COMPETING INTERESTS STATEMENT

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necrosis factor and interleukin 1. Earlier strategies in the treatment of sepsis inhibited each of these three proinflammatory factors but in all cases failed to improve outcomes². One potential explanation for the failure of these anti-inflammatory strategies is that as sepsis progresses, compensatory anti-inflammatory response syndrome may arise at different time points, resulting in immunosuppression and an anergic state⁵.

Rather than trying to target excessive immune stimulation, a more promising approach may be to inhibit the blood coagulation system, which is known to profoundly modulate the inflammatory response of the endothelium during sepsis⁶. In particular, activated protein C (APC) has proven beneficial in sepsis, producing a substantial reduction in the mortality rate of patients with late-stage disease⁷. APC has anti-inflammatory effects that have been proposed to be mediated by cleavage of protease-activated receptor 1 (PAR1), a G protein-coupled receptor^{8,9}. PAR1 is activated in a two-step process by proteases such as thrombin, plasmin, APC and matrix metalloprotease 1 (refs. 8–10–12): first the amino-terminal tethered ligand is ‘unmasked’ by proteolytic cleavage, then an intramolecular rearrangement allows the ligand and the receptor moieties to interact¹³. PAR1 is also critical in arterial thrombosis¹⁴ and is emerging as a chief regulator of angiogenesis during development^{15–17} and in malignant processes¹².

Support for the idea of a protective function for PAR1 in endothelial inflammation has been provided by a mouse model of ischemic stroke in which APC reduces damage by suppressing p53-initiated apoptosis by a mechanism dependent on PAR1 and endothelial protein C receptor^{18,19}. It has also been shown that APC loses its beneficial effects on survival in PAR1-knockout mice exposed to bacterial endotoxin (its toxic component consisting of lipopolysaccharide (LPS))²⁰. However, the main protease agonist of PAR1, thrombin, is also generated in large amounts during sepsis, thereby inducing PAR1-dependent cell contraction and barrier dysfunction of endothelial cells²¹ through Rho-dependent pathways^{22–24}. It is not clear whether activation of PAR1 signaling in endothelium by thrombin or APC protects against, exacerbates or has little net effect on the progression of sepsis.

Here we used a cell-penetrating peptide (‘pepducin’) approach to help delineate the effects of inhibiting versus activating PAR1 at specific stages during the progression of sepsis. Pepducins are lipid-conjugated, membrane-tethered peptides that can act as either agonists or antagonists of their cognate receptor^{4,25}. We found that pepducin antagonists and agonists based on the third intracellular loop of PAR1 had substantial beneficial or harmful effects on survival, vascular integrity and DIC in mice depending on the stage of sepsis. The effects of the PAR1-based pepducins were lost in PAR1-deficient mice, thus confirming the requirement for their cognate receptor. Notably, the protective effects of PAR1 activation required PAR2, a tethered-ligand receptor that is upregulated during inflammation^{26,27}. Transactivation of PAR2 signaling by PAR1 was enhanced by endotoxin-dependent recruitment of PAR1–PAR2 complexes to the endothelial surface, indicating that protease-receptor complexes are regulated entities that give rise to complex signaling phenotypes depending on the inflammatory state of the cell.

RESULTS

Effect of PAR1-based pepducins on the survival of septic mice

To gain insight into the function of endothelial PAR1 in sepsis, we administered PAR1-based pepducin agonists and antagonists²⁵ early and late after the initiation of septic peritonitis in mice. We confirmed that the PAR1 third intracellular loop-based pepducin agonist P1pal-13 (palmitate-AVANRSKKSRLF) was a full agonist for PAR1 but had no agonist activity for eight other endothelial G protein-coupled receptors that have been

linked to sepsis and inflammation (Fig. 1a). P1pal-12S (palmitate-RSLSSSAVANRS) was a full antagonist for PAR1 and did not inhibit any of the other tested G protein-coupled receptors in human embryonic kidney (HEK) cells (Fig. 1a) or the chemotactic responses of endothelial cells to the chemokines interleukin 8, SDF-1 α , sphingosine 1-phosphate (S1P), thromboxane, MCP1 and RANTES (data not shown).

We induced bacterial peritonitis by cecal ligation and puncture⁴ (CLP) and injected P1pal-12S subcutaneously either immediately after CLP or 4 h later. In untreated wild-type CF-1 (outbred) or C57BL/6 mice, the mortality rate from CLP-induced sepsis was 90–100% over a 7-day time period (Fig. 1b). Early administration of P1pal-12S (at 0 h) was highly beneficial to the survival of wild-type CF-1 and C57BL/6 mice. However, P1pal-12S lost its protective effect in the PAR1-knockout strain (*F2r*^{-/-} (called '*Par1*^{-/-}' here); Fig. 1b). Notably, delayed blockade (4 h) of PAR1 with P1pal-12S had no effect on the mortality of wild-type mice (Fig. 1b). Thus, early antagonism of PAR1 seems to be beneficial in septic mice, and P1pal-12S requires the presence of PAR1 to exert its beneficial effects.

Given that high doses of APC, a putative PAR1 agonist⁸, has salutary effects when given during severe sepsis^{7,28,29}, we tested whether P1pal-13 was beneficial when given at late times. Indeed, contrary to the effects of P1pal-12S, delayed administration (4 h) of P1pal-13 conferred a survival benefit of up to 80% to wild-type mice (Fig. 1b). Conversely, only 5% of the mice survived with immediate administration of P1pal-13 after CLP. The positive survival benefit of delayed P1pal-13 administration was lost in the *Par1*^{-/-} mice, in agreement with published data²⁵ indicating that the activity of the PAR1-based pepducin is dependent on the presence of its cognate receptor.

Effect of PAR1 agonists on lung vascular leakage

One of the most severe hemodynamic manifestations of sepsis is septic shock, characterized by increased cardiac output, a decrease in peripheral resistance and loss of intravascular fluid³⁰. We postulated that stage-specific blockade or activation of endothelial PAR1 would also affect the development of lung edema, a 'marker' of septic shock and an important contributing factor to the acute respiratory distress syndrome and increased mortality of septic patients³¹. First we assessed the effects of systemic stimulation of PAR1, PAR2 or PAR4 on lung vascular leakage in healthy mice. We injected PAR-selective peptide agonists into the internal jugular veins of CF-1 mice and assessed loss of barrier function as the accumulation of Evans blue dye in the lung interstitium. Infusion of the PAR1-selective agonist TFLLRN caused a 75% increase in lung vascular permeability in CF-1 mice, whereas neither the PAR2-selective agonist SLIGKV nor the PAR4-selective agonist AYPGKF had an effect (Fig. 2a). P1pal-13 caused a increase in lung vascular permeability similar to that caused by TFLLRN. Infusion of P1pal-12S or of the PAR4-antagonist pepducin P4pal-10 (ref. 32) did not affect vascular permeability.

Next we determined the effect of PAR1 activation or blockade on lung vascular permeability in mice that received direct injection of bacterial LPS. Intraperitoneal administration of a nonlethal dose of LPS (10 mg LPS per kg body weight (10 mg/kg)) caused the rapid appearance of lung interstitial fluid, reflecting a sixfold increase in lung vascular permeability over a 4-hour time period (Fig. 2b). Consistent with the effects on survival, early PAR1 blockade by P1pal-12S or late activation of PAR1 by P1pal-13 provided significant protection against lung vascular leakage (Fig. 2c). As noted with P1pal-12S, early blockade of thrombin with hirudin also produced significant inhibition of the LPS-induced fluid leakage (Fig. 2c).

We confirmed the effects of the PAR1-based pepducins on LPS-induced vascular leakage in the CLP model of sepsis. The development of lung fluid after CLP was biphasic (Fig. 2d).

The peak in lung vascular permeability occurred at 32 h and was approximately half that produced by the endotoxemia model. Early administration of P1pal-12S completely protected against lung fluid buildup, whereas late administration of P1pal-12S (at 8 h) exacerbated the vascular leakage (Fig. 2e). Delayed treatment with P1pal-13 protected against the further development of lung vascular leakage even when it was given 8 h after CLP. These data further suggest that late PAR1 activation, rather than its inhibition, provides beneficial effects against barrier dysfunction and leakage of fluid into the lung interstitium.

Late PAR1 agonism inhibits DIC

Endothelial cell activation by proinflammatory mediators and exposure of tissue factor after barrier disruption can lead to activation of the blood coagulation cascade. DIC is a severe sequela of sepsis characterized by the simultaneous appearance of microthrombi in small vessels and spontaneous bleeding. DIC is a result of the consumption of anticoagulant and procoagulant plasma proteins as well as a profound phenotypic switch in the endothelium from an antithrombotic state to a prothrombotic state³³. To assess the development of DIC, we measured the time course of the appearance of thrombin-antithrombin (TAT) complexes, the increase in fibrin degradation products (D-dimer) and platelet counts in septic mice over 2 d. TAT complexes increased as early as 2 h after CLP and remained increased thereafter (Supplementary Fig. 1a online). Platelet counts dropped by half over the first 8 h and the thrombocytopenia persisted for the remainder of the experiment (Fig. 3a). Circulating D-dimer amounts ‘spiked’ at 4 h, most likely as a consequence of the CLP surgery, and then increased by sixfold over the course of the second day (Fig. 3b).

To determine whether stage-specific inhibition and/or activation of PAR1 could affect the development of DIC, we injected PAR1-based pepducins at 0, 2, 4 or 8 h after CLP. We then killed the mice at 24 or 48 h after CLP to assess DIC parameters. Immediate treatment with P1pal-12S completely protected against thrombocytopenia and the appearance of D-dimer and TAT complexes (Fig. 3c,d and Supplementary Fig. 1b). However, P1pal-12S lost its protective effects against DIC when administered at later time points (2–8 h). In contrast, late administration of P1pal-13 protected mice from the development of DIC, as indicated by an increase in platelet counts and decreases in concentrations of D-dimer and TAT complexes. Thus, the effects of the PAR1-based pepducins on the indices of DIC correlated very well with survival outcome, which suggested that PAR1 may have either a direct or an indirect function in the development and progression of DIC in sepsis.

Protective effects of PAR1 require PAR2

The endothelium is a chief target of sepsis-induced events, and endothelial damage accounts for much of the pathology of septic shock. Actin-dependent contraction and loss of barrier function is driven by Rho-dependent phosphorylation of myosin light chain³⁴. The *in vivo* data reported above suggested that PAR1 may switch from a barrier-disrupting to a barrier-restoring receptor after exposure of the endothelium to endotoxin. To delineate the mechanism of the postulated LPS-induced PAR1 switch, we used an *in vitro* model of endothelial barrier function. We assessed barrier permeability as the leakage of Evans blue dye through a monolayer of EA.hy926 cells derived from human umbilical vein endothelial cells. Exposure of EA.hy926 cells to LPS caused an increase in permeability, with a maximum at 4 h (Fig. 4a). LPS-induced endothelial Rho-GTP activity preceded the loss of barrier function, with a peak at about 30 min that returned to near baseline by 240 min (Fig. 4b). Selective activation of PAR1 with TFLLRN led to twofold faster endothelial leakage than the LPS time course. After the peak at 30 min, the TFLLRN-induced leakage decreased by half by 4 h (Fig. 4a). P1pal-13 and the PAR1 and PAR2 agonist SFLLRN produced similar increases in permeability; however, P1pal-13 had a more prolonged effect. SLIGKV,

AYPGKF and P1pal-12S had no effect on the permeability of quiescent EA.hy926 monolayers.

We next simulated endothelial monolayers derived from EA.hy926 cells or from human pulmonary artery endothelial cells (HPAECs) with LPS and then with PAR-specific agonists to determine whether the effects on lung vascular permeability noted in the *in vivo* sepsis models could be recapitulated *in vitro*. Delayed treatment of LPS-stimulated EA.hy926 cells with SFLLRN or P1pal-13 at 2 h reversed 65–77% of the LPS-induced endothelial leakage (Fig. 4c,d), consistent with the *in vivo* data. Unexpectedly, however, delayed addition of TFLLRN did not restore barrier function to the LPS-treated endothelium (Fig. 4c). Unlike SFLLRN, which activates both PAR1 and PAR2, TFLLRN activates only PAR1. Therefore, the barrier-protective effects might require activation of PAR2. Indeed, delayed treatment of LPS-stimulated endothelium with the PAR2-selective peptide SLIGKV reversed 70–100% of the LPS-induced endothelial permeability (Fig. 4c,d). We obtained similar results with endothelial cells isolated from human pulmonary arteries (Supplementary Fig. 2a online).

To determine whether PAR2 was essential for the barrier-protective effects of PAR1, we suppressed expression of *Par2* in EA.hy926 cells and HPAECs by RNA-mediated interference (RNAi). After *Par2* was silenced, the barrier-restoring effects of SFLLRN, SLIGKV and P1pal-13 were abolished, in contrast to the effect of transfection with control small interfering RNA (siRNA) specific for luciferase (Fig. 4d,e). Silencing of *Par1* by RNAi also attenuated the late barrier-restoring effects of SFLLRN and P1pal-13. Thus, PAR1 is also essential for the protective effects of the PAR1 agonists noted above. To confirm the specificity of the *Par1* and *Par2* RNAi, we assessed calcium flux and did immunoblot analysis. Treatment of the endothelial cells with *Par1*-specific siRNA completely silenced PAR1 protein expression and prevented calcium mobilization in response to TFLLRN without blocking the calcium-mobilization response to SLIGKV (Fig. 4f,g). Conversely, *Par2*-specific siRNA suppressed 70% of PAR2 expression with little effect on PAR1 and prevented the calcium-mobilization response to SLIGKV without affecting the response to TFLLRN. Flow cytometry confirmed that 77% of PAR2 surface expression was suppressed by treatment with *Par2*-specific siRNA relative to treatment with luciferase-specific control siRNA (Supplementary Fig. 3a online).

The ability of P1pal-13 to mimic the effects of the agonists SFLLRN (PAR1 and PAR2) and SLIGKV (PAR2) but not the agonist TFLLRN (PAR1) suggested that P1pal-13 may also activate PAR2 in endothelial cells. One possible explanation is that P1pal-13 may transactivate PAR2 when expressed together with PAR1. PAR2 can be transactivated by thrombin-cleaved PAR1 in endothelial cells or when expressed together with PAR1 in fibroblasts, suggesting that PAR1 and PAR2 may exist as a heterodimer or oligomer³⁵. To test the possibility that P1pal-13 could transactivate PAR2, we expressed a ‘signaling-dead’ PAR1 mutant with transposition of two of the critical residues (D199R200) in the ‘DRY’ motif of transmembrane 3 (ref. 36; PAR1-RD) in the presence or absence of wild-type PAR2 in HEK cells. We found that PAR1-RD alone was unable to support chemotaxis toward a thrombin gradient (Fig. 5a). Likewise, when expressed alone, PAR2 was not able to produce a response to thrombin. However, when expressed together with PAR1-RD, PAR2 was able to produce chemotaxis toward a thrombin gradient. This result is consistent with those of published studies showing that thrombin-cleaved PAR1 is able to donate its tethered ligand to transactivate PAR2 (ref. 35). Like thrombin, P1pal-13 was able to act as a chemotactic factor for cells expressing wild-type PAR1 but not for those expressing PAR2 or PAR1-RD (Fig. 5b). However, expression of PAR1-RD together with PAR2 produced full chemotactic activity toward P1pal-13. We repeated these experiments with the well characterized PAR1-null human breast carcinoma cell line MCF7 (ref. 37). The results obtained with the MCF-7 cells were nearly identical to those obtained with HEK cells

(Supplementary Fig. 2b). Thrombin and P1pal-13 were not able to stimulate chemotactic migration of MCF-7 cells expressing PAR1-RD or wild-type PAR2 unless the two receptors were expressed together. These data are consistent with a PAR1–PAR2 transactivation mechanism whereby PAR1 can either donate its tethered ligand to PAR2 on the outside or support allosteric activation of PAR2 by P1pal-13 on the cytoplasmic side. P1pal-12S blocked chemotaxis toward thrombin elicited by wild-type PAR1 but did not inhibit PAR2-dependent chemotaxis elicited either by SLIGKV or by the tethered ligand donated by PAR1-RD (Fig. 5c and Supplementary Fig. 2b). Thus, P1pal-12S does not inhibit the activity of PAR2 even when transactivated by PAR1.

The PAR1–PAR2 transactivation mechanism proposed above would indicate that loss of PAR2 would also eliminate the protective effects of P1pal-13 on survival, DIC and lung vascular leakage in septic mice. PAR2, however, should not be required for the early damaging effects of PAR1 activation. To test these possibilities *in vivo*, we compared the effects of PAR1-agonist versus PAR1-antagonist pepducins in septic mice lacking PAR2. Late treatment with P1pal-13 had no salutary effect on the survival of *Par2*^{−/−} mice subjected to CLP and may have produced a trend toward slightly less survival ($P = 0.14$; Fig. 5d). However, early treatment of the *Par2*^{−/−} mice with P1pal-12S was still able to confer a ‘benefit’ to the mortality rate relative to treatment with vehicle ($P = 0.042$). Next we determined whether PAR2 deficiency also abolished the beneficial effects of P1pal-13 on coagulation and lung barrier function. Indeed, loss of PAR2 eliminated the protective effects of late treatment with P1pal-13 on concentrations of TAT complexes, platelet counts and lung vascular permeability but did not prevent the protective effects conferred by early treatment with P1pal-12 (Fig. 5e–g). These survival, coagulation and lung vascular leakage data are collectively in agreement with the *in vitro* endothelial barrier function data and suggest that PAR2 is required for the late protective effects of P1pal-13 but is not required for the protective effects afforded by early inhibition of PAR1.

Protective effects of thrombin and APC on endothelium

Early blockade of thrombin with hirudin or of PAR1 with P1pal-12S was effective in protecting against lung vascular permeability in the septic mice, which suggested that thrombin cleaves and activates endothelial PAR1 at early stages in the disease process. Large amounts of thrombin were also produced at later stages of CLP-induced sepsis (Supplementary Fig. 1), which indicated that endothelial PAR1 might be continuously exposed to thrombin. To begin to parse the stage-specific effects of thrombin, we compared the effects of thrombin on quiescent versus endotoxin-stimulated endothelium. Treatment of quiescent endothelial monolayers with LPS or thrombin caused the cells to contract and lose cell-cell contacts, as assessed by staining of filamentous actin with fluorescein isothiocyanate–phalloidin (Fig. 6a), consistent with published studies²². However, delayed treatment with 0.3–3 nM thrombin at 2 h after exposure to LPS protected against barrier disruption, as indicated by both phalloidin staining and leakage of Evans blue dye (Fig. 6a,b).

To further investigate whether the barrier-repair activity of late treatment with thrombin required PAR2, we transfected cells with siRNA specific for *Par2*, *Par1* or luciferase control. Pretransfection of the LPS-damaged EA.hy926 or HPAEC monolayers with *Par2*-specific siRNA completely abolished the protective effects of late treatment with thrombin (Fig. 6c,d). Consistent with the peptide agonist experiments reported above, silencing of *Par1* by RNAi also ablated the effects of late treatment with thrombin (Fig. 6c,d). Thus, these data confirm that both PAR1 and PAR2 are required for the reparative effects of late treatment with thrombin on LPS-damaged endothelium.

APC has also been shown to mediate barrier stabilization by activation of PAR1 through pathways dependent on the receptor for S1P (S1P₁)^{38,39}. Contrary to results obtained with thrombin or P1pal-13, a high concentration of APC (180 nM) had little effect on the morphology or intercellular contacts of quiescent endothelial cells (Fig. 6a). When given simultaneously with LPS, 180 nM APC produced slight protection against the barrier-disrupting effects of LPS, whereas in the same conditions, thrombin exacerbated the effects of LPS (Fig. 6b). Late treatment (at 2 h) of LPS-stimulated endothelium with a high concentration of APC conferred a protective effect, albeit with about 0.1–2% the potency of thrombin (Fig. 6d). The early and late barrier-protective effects of 180 nM APC were abolished by silencing with siRNA specific for either *Par1* or *Par2* (Fig. 6c,d). These data collectively indicate that the protective effects of APC on barrier function required both PAR1 and PAR2 but they were less dependent on the pre-existing state of the endothelium than the effects of thrombin were.

A thrombin signaling switch in LPS-stimulated endothelium

The ability of PAR1 to switch from a barrier-disruptive to a barrier-protective receptor indicated that opposing G protein signaling pathways were being engaged by PAR1 as the endothelium changed from a quiescent to a stimulated state. Two candidate cytoskeletal-altering pathways were the G₁₂-G₁₃-Rho-MLC and the G_i-Rac axes, which control cell contraction and spreading, respectively⁴⁰. We postulated that in quiescent endothelium or during initial exposure to endotoxin, PAR1 disrupts barrier integrity through a G₁₂-G₁₃-Rho-dependent pathway; then, after exposure to endotoxin, PAR1 switches its signaling to activate G_i signaling pathways, which mediate barrier repair by a Rac mechanism. We found that in quiescent HPAECs, thrombin- and TFLLRN-dependent barrier disruption was completely blocked by a Rho inhibitor but not by blockade of G_i (Fig. 7a). As reported above, activation of PAR2 by SLIGKV had no effect on the endothelial permeability of the quiescent HPAECs (Fig. 7a). We obtained similar results with EA.hy926 cells (Supplementary Fig. 2c). These data are consistent with a mechanism whereby initial endothelial leakage occurs through activation of PAR1-Rho pathways by thrombin independently of PAR2.

We tested whether blockade of G_i would prevent the late protective effects of thrombin on LPS-stimulated endothelium. LPS stimulation of endothelial leakage in both HPAECs and EA.hy926 cells was completely blocked by the Rho inhibitor but not by the G_i inhibitor (Fig. 7b,c). Notably, the barrier-restoring effect of thrombin was abrogated by blockade of G_i signaling (Fig. 7b,c). However, selective activation of PAR1 with TFLLRN lacked the ability to confer barrier-restoring activity to the LPS-stimulated endothelium and was unaffected by blockade of G_i. Thus, ‘downstream’ PAR1 signaling in endotoxin-stimulated cells differs substantially when activated by the PAR1-selective peptide or thrombin. The barrier-protective effect caused by late activation of PAR2 with SLIGKV was completely blocked by pertussis toxin, demonstrating that PAR2 exerts its protective effects through a G_i pathway (Fig. 7b,c). Therefore, thrombin signaling seems to resemble PAR2 signaling in LPS-stimulated cells, consistent with the idea that PAR1 cleavage in endotoxin-stimulated cells induces PAR2 signaling by receptor transactivation.

Thrombin stimulation of Rac requires PAR2

We transfected cells with *Par2*-specific siRNA or luciferase-specific control siRNA to determine whether PAR2 participates in thrombin signaling to Rac and Rho. In the absence of LPS, quiescent endothelial cells treated with either *Par2*-specific or luciferase-specific siRNA had small basal amounts of Rho-GTP but large basal amounts of Rac-GTP (Fig. 7d,e). Stimulation with LPS, thrombin, TFLLRN or P1pal-13 caused a substantial increase in Rho-GTP and suppression of Rac-GTP in endothelial cells treated with luciferase-specific

siRNA, but stimulation with SLIGKV or P1pal-12S did not (Fig. 7d). However, treatment of LPS-stimulated endothelium with the various agonists led to a reversal of the effects in cells treated with luciferase-specific siRNA: Rac-GTP remained high in response to delayed treatment with thrombin, SLIGKV or P1pal-13, whereas Rho-GTP activity was suppressed (Fig. 7d). Silencing of *Par2* by RNAi had no effect on the initial effects on Rho-GTP and Rac-GTP induced by LPS, thrombin or P1pal-13 (Fig. 7e). As expected, 'knockdown' of *Par2* by RNAi inhibited the Rac-activating and Rho-suppressing effects of delayed treatment with SLIGKV on the LPS-stimulated endothelium (Fig. 7e). Likewise, silencing of *Par2* prevented the Rac-activating and Rho-suppressing effects of delayed treatment with thrombin or P1pal-13 on the LPS-stimulated endothelium. Therefore, the ability of thrombin or P1pal-13 to activate the barrier-protective activity of Rac-GTP and suppress the barrier-disrupting activity of Rho-GTP is dependent on the presence of PAR2.

LPS relocates PAR1–PAR2 complexes to the cell surface

The observed switch in thrombin signaling was consistent with a transactivation mechanism whereby the tethered ligand of thrombin-cleaved PAR1 directly activates PAR2 in endotoxin-stimulated endothelium. The transactivation mechanism would require that PAR1 and PAR2 come into close contact with each other on the cell surface. To detect PAR1–PAR2 complexes, we used photobleaching confocal fluorescence resonance energy transfer (FRET) microscopy of live endothelial cells with a construct of carboxy-terminally truncated PAR2 with cyan fluorescent protein attached in-frame to PAR2 residue 372 (PAR2 Δ 372-CFP) as the fluorescent donor and a construct of carboxy-terminally truncated PAR1 with yellow fluorescent protein attached in-frame to PAR1 residue 377 (PAR1 Δ 377-YFP) as the fluorescent acceptor¹⁴. A FRET signal occurs when fluorescent donors and acceptors are in close molecular proximity (less than 100Å apart). We noted a punctate FRET signal in the cytoplasm when PAR1 Δ 377-YFP and PAR2 Δ 372-CFP were expressed together in untreated endothelial cells (Fig. 7f) but not in cells individually transfected with either PAR1 Δ 377-YFP or PAR2 Δ 372-CFP (Supplementary Fig. 4a online). After the addition of LPS, there was considerable relocation of the PAR1–PAR2 FRET signal to the cell periphery and plasma membrane by 1 h (Fig. 7f and Supplementary Fig. 4b,c). For independent confirmation that LPS caused PAR1–PAR2 complexes to move to the plasma membrane, we did chemical crosslinking of untreated versus LPS-treated endothelial cells. Endothelial PAR1 appeared as two clusters of bands migrating at 62–85 kilodaltons and 40 kilodaltons (Supplementary Fig. 4d), in agreement with the glycosylated PAR1 dimer and monomer described before^{11,14}. The corresponding immunoblot for endothelial PAR2 showed a more heterogeneous distribution of bands, with a major species clustered near 55 kilodaltons, as reported before⁴¹. The addition of the hydrophilic, non-cell-permeable lysine crosslinking agent BS3 had no effect on the mobility of the various PAR1 and PAR2 species from quiescent endothelium. However, the addition of BS3 to LPS-stimulated endothelium led to the appearance of a species of high apparent molecular mass (190 kilodaltons) that was stained by antibodies to both PAR1 and PAR2 (Supplementary Fig. 4d). These data provide independent evidence that LPS induces PAR1–PAR2 complexes to appear on the surface of endothelium, where the proteolytically cleaved, tethered ligand of PAR1 can access and activate PAR2 (Supplementary Fig. 5).

DISCUSSION

Using pepducin technology, we have provided evidence here that activation of PAR1 is harmful during early phases of sepsis in mice but becomes beneficial at later stages in a PAR2-dependent way. Our data are consistent with a mechanism whereby the time-dependent switch in the inflammatory functions of endothelial PAR1 is dependent on the ability of PAR1 to transactivate PAR2 signaling. Endotoxin first induces barrier-disrupting

effects on endothelium, causing initial vascular damage and activation of the blood coagulation system. The thrombin generated early activates PAR1–G₁₂–G₁₃ signaling pathways, causing additional Rho-dependent cell contraction, perpetuation of the vascular damage and DIC. However, by a negative feedback loop, stimulation of the endothelium with endotoxin induces relocalization of PAR1–PAR2 complexes to the plasma membrane, whereupon PAR1 switches its signaling by transactivation of PAR2–G_i–Rac protective pathways that culminate in barrier repair and attenuation of DIC.

We also found that high concentrations of APC had little protective effect on endothelial barrier function unless they were given after the endothelium was exposed to endotoxin. Likewise, activation of PAR1 with P1pal-13 was beneficial only when it was administered at later times during the progression of sepsis or endotoxemia. This finding is consistent with published *in vitro* studies showing that APC restores endothelial barrier function through PAR1–S1P₁ pathways^{38,39}. Conversely, early blockade of PAR1 with P1pal-12S was beneficial at early stages of sepsis. The time-dependent switch of PAR1 from an ‘exacerbating’ receptor to a protective receptor is consistent with the observation that PAR1 deficiency confers no net survival benefit in endotoxemia models of sepsis^{42,43}, as also noted in our CLP model. Because PAR1 has opposing, temporally controlled functions during the progression of sepsis, genetic deficiency in PAR1 may not provide net protection if PAR1 is not available to transactivate PAR2 barrier-repair pathways.

Our data have indicated that PAR2 may have a mainly protective function in sepsis. Similarly, it has been shown that PAR2 agonism exerts anti-inflammatory effects in rodent models of LPS-induced lung injury^{44,45}. PAR2 activation has also been shown to protect against reperfusion injury after prolonged ischemia in rat hearts⁴⁶. However, activating PAR2 has been shown to have damaging rather than protective effects in animal models of arthritis, neurogenic pain and inflammation^{47,48}. Our idea that PAR1 can confer protective effects through transactivation of PAR2 would suggest that the signaling outputs of PAR1 and PAR2 are entwined and need to be examined as a potential functional unit in each of these inflammatory conditions. Furthermore, the data suggest that PAR1 and PAR2 might demonstrate their beneficial, anti-inflammatory phenotypes in defined ‘time windows’ after exposure to proinflammatory stimuli.

The functional interaction detected between PAR1 and PAR2 in these sepsis studies adds to the precedent of the formation of receptor complexes or ‘cofactoring’ that occurs with many members of the PAR family. Transactivation of PAR2 by PAR1 has been demonstrated in COS-7 fibroblast cells coexpressing PAR2 and a mutant PAR1 that can be cleaved but cannot signal³⁵. Intermolecular activation of PAR2 by the thrombin-generated PAR1 ligand did not require prior treatment of the fibroblasts with LPS or other stimuli. This result suggests that PAR1–PAR2 complexes may perhaps in some cases be localized to the cell surface by overexpression. Notably, treatment of endothelial cells with LPS, tumor necrosis factor or interleukin 1 results in a five- to tenfold upregulation of PAR2 mRNA and protein expression²⁶, which could also favor the formation of PAR1–PAR2 complexes in proinflammatory conditions. Transactivation of PAR2 by PAR1 may also be important for cancer cell motility and metastasis. Studies of melanoma cells have indicated that stimulation of cell motility by thrombin requires not only the activation of PAR1 but also the simultaneous activation of PAR2 (ref. 49). Constitutive formation of heterodimers or oligomers of PAR1 and PAR4 has been demonstrated in human platelets and fibroblasts, in which the high-affinity thrombin receptor PAR1 assists in the cleavage of the low-affinity thrombin receptor PAR4 (ref. 14). Therefore, PAR1 may control the activity of other PARs either by delivering its tethered ligand, for PAR2, or by delivering thrombin when in complex with PAR4. This unique ability of PARs to react to extracellular proteases, combined with their ability to transactivate each other, may confer a greatly expanded range

of phenotypes to cells exposed to the rapidly changing environment encountered during severe inflammatory conditions such as sepsis.

METHODS

Reagents

The PAR peptide ligands SFLLRN, TFLLRN, SLIGKV, AYPGKF and the pepducins P1pal-12S and P1pal-13 were synthesized with carboxy-terminal amides by standard fluorenylmethoxycarbonyl solid-phase methods²⁵. Pepducins were purified to 95–98% by C₄ reversed-phase HPLC and were dissolved in dimethyl sulfoxide (DMSO). Thrombin and APC were from Haematologic Technologies; LPS endotoxin (*Escherichia coli* 0111:B4), Evans blue dye and recombinant hirudin were from Sigma Aldrich; BS3 (bis(sulfosuccinimidyl) suberate) was from Pierce; and lipofectamine and oligofectamine were from Invitrogen. *Par1*-specific siRNA (5'-AAGGCUACUAUGCCUACUACU-3') and firefly luciferase-specific siRNA (5'-CGTACGCGGAATACTTCGA-3') were from Dharmacon; *Par2*-specific siRNA (5'-AGGAAGAAGCCUUAUUGGU-3') was from Ambion. Polyclonal antibody to the PAR2 ligand region of residues S38LIGKVDGTSHVTGKGV54C (SLIG-Ab) was generated by published methods¹¹ and was purified from rabbit antisera with a S38–V54C peptide–Sepharose 4B affinity column. As a specificity control, SLIG-Ab had no detectable reactivity to PAR1 expressed in N55 MCF7-derived cells⁴ with high expression of PAR1 (Supplementary Fig. 3b). Monoclonal antibody ATAP2 to PAR1 was from Santa Cruz Biotechnology, and fluorescein isothiocyanate-conjugated secondary antibodies were from Zymed. Enzyme-linked immunoassay (ELISA) kits to assess D-dimer were from Diagnostica Stago, and TAT complex ELISA kits were from Behring.

Cell culture

HPAECs were from Cambrex; EA.hy926 cells are a transformed human umbilical vein endothelial cell line⁵⁰. Cells were grown in EGM-2 medium (Cambrex) supplemented with 10% (vol/vol) endotoxin-free FBS (Gibco). For transient transfection with Myc-tagged Rac DNA, lipofectamine and 20 µg DNA per 100-mm dish were used. Experiments were done 48 h after Myc-Rac transfection. Oligofectamine was used for transfection of siRNA (20 µM per plate) and experiments were done 48 h after transfection. The siRNA transfection efficiency for permeability assays was determined by transfection with green fluorescent protein–pcDNA3 (2 µg per 100-mm plate) and values were normalized to the number of green fluorescent protein-positive cells.

Endothelial permeability assays

HPAECs and EA.hy926 cells were plated on polycarbonate Transwell membranes with a pore size of 3 µm (Corning) in EGM-2 medium supplemented with 10% (vol/vol) FBS. After cells reached confluence, the cell-coated membranes were placed in a dual-chamber system and were treated with LPS (1 µg/ml) and/or PAR agonists and inhibitors. In some experiments, LPS was added 2 h before the peptides or proteases. After incubation, growth media was removed and DMEM containing Evans blue dye (30 mg/ml) was added to the upper chamber and PBS was added to the lower chamber. After 15 min of incubation, Evans blue was quantified in the lower well by measurement of absorbance at 650 nm.

Actin staining of endothelial cells

Endothelial cells were plated onto tissue culture chamber slides and were grown to confluence. After reaching confluence, cells were treated with 0.2% (vol/vol) DMSO (vehicle), 0.3 nM thrombin, 0.3 µM P1pal-13 or 180 nM APC in PBS. In another set of

experiments, cells were treated with LPS (1 $\mu\text{g/ml}$) plus PAR agonists or agonists added 2 h after the initial LPS treatment. At 4 h, cells were fixed and stained with fluorescein isothiocyanate–conjugated phalloidin.

Rho and Rac assays

EA.hy926 cells were transfected with Myc-tagged Rac 48 h before the assays. Rho-GTP was precipitated with glutathione *S*-transferase (GST)–rhotekin–reduced glutathione–agarose beads and Myc-Rac-GTP was precipitated with GST–PAK–reduced glutathione–agarose beads as described⁵¹. Rho-GTP and Myc-Rac-GTP were then quantified by immunoblot analysis with monoclonal antibody to RhoA (26C4) and monoclonal antibody to Myc (9E10), respectively; a portion of the endothelial cell lysates was also analyzed by immunoblot with monoclonal antibody to Rac (C14) for detection of total Rho and Myc-Rac, as a loading control (all three antibodies from Santa Cruz Biotechnology).

Crosslinking of cell surface receptors

Confluent EA.hy926 cells were stimulated for 4 h at 37 °C with LPS (10 $\mu\text{g/ml}$) or PBS in 100-mm plates. BS3 was then added at a concentration of 1 mM. Crosslinking was ‘quenched’ after 30 min with 20 mM Tris-HCl, pH 7.2, and 20 mM ethanolamine. Cells were then lysed with lysis buffer (0.1% (vol/vol) Triton X-100, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF and 1 mM phenylmethylsulfonylfluoride) and were analyzed by immunoblot with ATAP2 (antibody to PAR1) and SLIG-Ab (antibody to PAR2).

FRET microscopy

PAR2 Δ 372-CFP (donor) and PAR1 Δ 377-YFP (acceptor) were transfected together into EA.hy926 cells with LipofectAmine. After 24 h, transfected cells were treated with LPS (1 $\mu\text{g/ml}$) or vehicle. A Leica TCS SP2 instrument was used for confocal photobleaching FRET microscopy, with excitation with argon lasers (458 nm, 5 mW; 514 nm, 20 mW). Donor emission before acceptor photobleaching was collected at 460–500 nm and acceptor emissions were collected at 520–550 nm. The acceptor was photobleached with several pulses of irradiation at 514 nm. Donor and acceptor emissions were collected again after photobleaching. The final FRET efficiency image was calculated with the following algorithm: [donor FRET (after) – donor FRET (before)] / donor FRET (after).

Animals

Male and female CF-1 or C57BL/6 mice (6–8 weeks old) were from Charles River Laboratories. *Par1*^{−/−} and *Par2*^{−/−} mice on a C57BL/6 background (backcrossed at least ten generations) were generated as described^{52,53}. Animal experiments were done in accordance with guidelines of the US National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Tufts–New England Medical Center. CLP was done as described⁵⁴. Blood for platelet counts and ELISA was collected by cardiac puncture. Concentrations of fibrin D-dimer and TAT complexes in plasma were measured by ELISA according to the manufacturer’s instructions. Platelets were counted in a Hemavet 850 as described⁴. For quantification of lung vascular permeability, the internal jugular vein was catheterized and 100 μl of Evans blue dye was injected (20 mg/kg). After dye was allowed to circulate for 30 min, mice were killed and their lungs were flushed five times with PBS. Lungs were then weighed and Evans blue was extracted overnight at 70 °C with formamide and was measured by absorbance at 650 nm as described⁵⁵.

Statistical analyses

The Mann-Whitney U-test after the Kruskal Wallis analysis of variance was used for statistical analysis. The Kaplan-Meier log-rank test was used for survival analyses. *P* values of less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effects of PAR1 agonist and antagonist pepducins on the survival of septic mice. **(a)** Migration of HEK cells transiently transfected with cDNA encoding human G protein-coupled receptors (horizontal axis) toward RPMI medium plus 0.5% BSA alone or containing the following agonists: TFLLRN (10 μ M), for PAR1; SLIGKV (10 μ M), for PAR2; AYPGKF (100 μ M), for PAR4; interleukin 8 (50 nM), for CXCR1 and CXCR2; SDF-1 α (100 nM), for CXCR4; S1P (100 nM), for S1P₁ and S1P₃; or RANTES (20 ng/ml), for CCR5 (ref. 4). P1pal-12S (2 μ M) or P1pal-13 (1 μ M) was added at time 0 and migration in the Transwell apparatus (8- μ m pore) was stopped after 20 h. The chemotactic index (mean $\pm$ two s.e.m.) is the ratio of directed migration to random migration (RPMI); $n = 5-7$

wells per data point. *, $P < 0.05$. **(b)** Survival of wild-type CF-1 mice ($n = 10-15$), wild-type C57BL/6 mice ($n = 12$) or *Par1*^{-/-} C57BL/6 mice ($n = 8-12$) subjected to CLP, then given vehicle, P1pal-12S or P1pal-13 (2.5 mg/kg) subcutaneously immediately (0 h) or 4 h later; mice then received a maintenance dose of pepducins (1 mg/kg) subcutaneously every 24 h until day 6. P values, pepducin versus vehicle (Kaplan-Meier comparisons made with the nonparametric log-rank test). Data are representative of three experiments.

Figure 2.

Effect of activation or inhibition of PAR1 on lung vascular permeability in septic mice. **(a)** Lung vascular permeability after injection of CF-1 mice ($n = 5$) with peptide (5 mg/kg) or vehicle (Veh; 100 μ l of 80% PBS and 20% DMSO) over 1 min, quantified at 45 min as the accumulation of Evans blue dye in lung interstitium over 30 min and presented as peptide treatment relative to vehicle treatment (set as 1). **(b)** Lung vascular permeability after intraperitoneal injection of LPS (10 mg/kg; nonlethal dose) or buffer (PBS), measured as described in **a** and presented as LPS treatment relative to buffer treatment ($n = 6$ mice). **(c)** Lung vascular permeability after injection of LPS (10 mg/kg, intraperitoneally), then pepducins (5 mg/kg, subcutaneously) or hirudin (10 mg/kg, intraperitoneally) either immediately (0 h) or 2 h later, measured as described in **a** at 4 h ($n = 3$ mice). **(d)** Lung vascular permeability after the induction of sepsis by CLP at time 0, assessed as described in **a** from 2 h to 48 h. **(e)** Lung vascular permeability in mice subjected to CLP and injected with P1pal-12S or P1pal-13 (each 2.5 mg/kg, subcutaneously), assessed as described in **a** at 24 h. *, $P < 0.05$ (**a,c,e**). Data are representative of three experiments.

Figure 3.

Treatment of septic mice with PAR1-based pepducins inhibits DIC. **(a)** Time course of thrombocytopenia after CLP of CF-1 mice ($n = 6$) at time 0; platelets were counted in platelet-rich plasma. **(b)** ELISA of the time course of the changes in plasma concentrations of D-dimer after CLP of CF-1 mice ($n = 6$). **(c)** Thrombocytopenia in mice ($n = 5$) injected once with pepducins (2.5 mg/kg, subcutaneously) at 0, 2, 4 or 8 h after CLP; platelets were counted 24 h after CLP. *, $P < 0.05$. **(d)** Formation of D-dimers in CF-1 mice ($n = 6$) treated with P1pal-12S (2.5 mg/kg) or P1pal-13 (2.5 mg/kg) either immediately (0 h) or 8 h after CLP and assessed 48 h later. Sham, laparotomy only. *, $P < 0.05$. Data are representative of three experiments.

Figure 4.

The beneficial effects of PAR1 agonists on endothelial barrier function require PAR2. **(a)** Permeability of quiescent EA.hy926 monolayers grown to confluence on Transwell membranes (3 μ m pore), then stimulated with LPS (1 μ g/ml), TFLLRN (10 μ M), SFLLRN (100 μ M), SLIGKV (100 μ M), AYPGKF (100 μ M), P1pal-13 (0.3 μ M) or P1pal-12S (0.3 μ M); after 30–240 min of incubation, Evans blue (30 mg/ml in DMEM) was added to the upper well and leakage (15 min) into the bottom well was measured as absorbance at 650 nm. Results are presented as stimulated relative to unstimulated control. **(b)** Immunoblot analysis of the time course of LPS activation of Rho in EA.hy926 monolayers grown to confluence and treated with LPS (time, above lanes); cell lysates were analyzed for active Rho-GTP and total Rho. **(c)** Permeability of confluent endothelial monolayers stimulated with LPS (1 μ g/ml) and then treated with peptide agonists (concentrations as in **a**) or 0.2% DMSO vehicle immediately after LPS challenge (0 h) or 2 h later; permeability was measured at 4 h (0% is the permeability of quiescent cells; 100% is the permeability of LPS-stimulated cells). *, $P < 0.05$. **(d,e)** Permeability of EA.hy926 cells **(d)** or HPAECs **(e)** grown on membranes (3- μ m pore) and transfected with siRNA specific for *Par1*, *Par2* or luciferase (Luci) control; after 48 h, cells were stimulated with LPS and then peptide agonists (100 μ M) were added 2 h later and permeability was measured at 4 h. *, $P < 0.05$. **(f)** Intracellular calcium mobilization in EA.hy926 cells transfected with siRNA specific for *Par1*, *Par2* or luciferase control; after 48 h, cells were ‘lifted’ with 1 mM EDTA in PBS and loaded with the dye Fura-2AM, and calcium mobilization (Ca^{2+}) was monitored as the ratio of fluorescence excitation intensity at 340 to that at 380 nm, as described⁵⁶. AU, arbitrary units. **(g)** Immunoblot (IB) of lysates of siRNA-transfected cells (48 h), analyzed with monoclonal antibodies ATAP2 (anti-PAR1) and SLIGK (anti-PAR2). Data are representative of three **(a,c–f)**, or four **(b,g)** independent experiments.

Figure 5.

The protective effects of the PAR1-agonist pepducin on the survival, DIC and vascular permeability of septic mice require PAR2. (a–c) Migration of HEK cells left untransfected (–) or transiently transfected with pcDEF3 vector, PAR1 (wild-type), PAR1-RD or PAR2 (wild-type) toward chemotactic gradients of thrombin (T; 0.3 nM), P1pal-13 (1 μM), SLIGKV (10 μM), P1pal-12S (3 μM) or RPMI, for 24 h in a Transwell microchemotaxis apparatus (8-μm pore), presented as described in Figure 1a. * $P < 0.01$. (d) Survival of *Par2*^{-/-} C57BL/6 mice ($n = 17–20$) subjected to CLP and then injected subcutaneously with vehicle, P1pal-12S (2.5 mg/kg) or P1pal-13 immediately (vehicle or P1pal-12S) or 4 h later (P1pal-13); mice then received pepducin (1 mg/kg) subcutaneously every 24 h until day 6.

(e) ELISA of TAT complex concentrations in the blood of *Par2*^{-/-} mice subjected to CLP, then injected subcutaneously with P1pal-12S or P1pal-13 (2.5 mg/kg) either immediately or 4 h later, and assessed 48 h after CLP. (f) Thrombocytopenia in *Par2*^{-/-} mice subjected to CLP, then injected subcutaneously with pepducins (2.5 mg/kg) or vehicle immediately or 4 h later; platelets were counted 24 h after CLP. (g) Lung vascular permeability in *Par2*^{-/-} mice subjected to CLP, then injected subcutaneously with P1pal-12S or P1pal-13 (2.5 mg/kg) immediately or 4 h later; permeability was assessed at 24 h and is presented relative to that of vehicle-treated mice (set as 100%). *, *P* < 0.05. Data are representative of six (a–c) or three (d–g) experiments.

Figure 6.

The endothelial barrier–restoring activity of thrombin and APC requires PAR1 and PAR2. (a) Barrier integrity of EA.hy926 cells grown to confluence on Transwell membranes (3- μ m pore), then exposed to LPS (1 μ g/ml) or 0.2% DMSO vehicle (–) at time 0 (right margin), and then treated with vehicle, thrombin (0.3 nM), APC (180 nM) or P1pal-13 (0.3 μ M) either at the time of LPS challenge or 2 h later (left margin); at 4 h, all cells were stained with fluorescein isothiocyanate–phalloidin and analyzed for retraction or spreading. Original magnification, $\times 160$. (b) Permeability of confluent EA.hy926 cells stimulated with LPS (1 μ g/ml) and then treated with thrombin (Thr) or APC immediately (0 h) or 2 h later and assessed at 4 h. Results are presented relative to unstimulated control. (c,d) Permeability of EA.hy926 cells (c) or HPAECs (d) transfected with siRNA specific for *Par1*, *Par2* or luciferase; 2 d later, cells were stimulated with LPS (1 μ g/ml), treated with thrombin or APC 2 h later, and assessed at 4 h. *, $P < 0.05$. Data are representative of three independent experiments.

Figure 7.

PAR1–PAR2 switching of Rac and Rho signaling in endotoxin-stimulated endothelium. **(a)** Permeability of quiescent confluent HPAECs treated overnight with DMEM containing buffer (PBS), C3 transferase (100 μ g/ml) or pertussis toxin (PTX; 100 ng/ml), then washed and exposed to thrombin (0.3 nM), TFLLRN (10 μ M), SLIGKV (100 μ M), P1pal-12S (0.3 μ M) or P1pal-13 (0.3 μ M); permeability was assessed as the leakage of Evans blue into the lower wells. **(b,c)** Permeability of HPAECs **(b)** or EA.hy926 cells **(c)** pretreated with C3 transferase or PTX and stimulated with LPS (1 μ g/ml); PAR agonists or antagonists were added 2 h later and endothelial permeability was assessed at 4 h. **(d,e)** Immunoblot of EA.hy926 cells transfected with luciferase-specific siRNA **(d)** or *Par2*-specific siRNA **(e)**; 2 d later, cells were exposed for 5 min to LPS or buffer (–), then thrombin (0.3 nM), SLIGKV (100 μ M), TFLLRN (10 μ M), P1pal-12S (0.3 μ M) or P1pal-13 (0.3 μ M) was added and, at 2 h, cells were lysed and activated Rho-GTP was precipitated with GST-Rhotekin beads and Rho-GTP was quantified by analysis with RhoA-Ab. For the corresponding Rac assays, EA.hy926 cells were pretransfected with Myc-tagged Rac; activated Rac was precipitated with GST-PAK beads and was quantified by immunoblot analysis with antibody to Myc. Total Rho and Rac, immunoblot of whole-cell lysates (loading control). Intensity (below lanes) is relative to negative control, set as 1. **(f)** Confocal photobleaching FRET microscopy of EA.hy926 endothelial cells transfected with PAR2 Δ 372-CFP (donor) and PAR1 Δ 377-YFP (acceptor). Blue indicates low FRET efficiency; red (arrowheads) indicates high FRET efficiency. Original magnification, $\times 400$. Data are representative of three experiments **(a–c)**; four to five experiments with similar results **(d,e)**; or 10–20 cells per condition in three independent experiments **(f)**.