The evolving plasticity of coagulation protease-dependent cytoprotective signalling

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Summary
Coagulation proteases control cellular homeostasis beyond haemostasis. While the role of coagulation proteases in regulating vascular healing and thrombosis is well established, the mechanism underlying the receptor-dependent regulation of cellular function remains incompletely understood. In particular, the opposing effects of the protease-activated receptor 1 (PAR-1), dependent on the activating proteases thrombin or activated protein C generated a conundrum researchers only recently have begun to decipher. The net-effect (cellular perturbation vs. cellular protection) depends on co-receptors involved, the concentration of the activating protease, the temporal context of receptor activation, and a dynamic process of receptor rearrangement upon receptor activation. The latter scenario recruits receptors to a cytoprotective signalling pathways. Recent insights into these mechanisms are summarized in this article.

Keywords
Protease activated receptor, protein C, thrombin, cytoprotection, biased signalling

Schlüsselwörter
Protease-aktivierbarer Rezeptor, Protein C, Thrombin, Zellprotektion

Zusammenfassung

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Activated protein C and cytoprotection

Beyond its function in haemostasis the blood coagulation system has an increasingly recognized function for cellular homeostasis particularly in the body’s defense system (Fig. 1). The cellular effects are mediated via receptor-dependent mechanisms. The identification of protease-activated receptors (PAR) initiated experimental efforts to decipher the signalling mechanism through which coagulation proteases modulate cellular functions. These efforts were hampered by apparently paradoxical findings, e. g. distinct effects of the protease thrombin in various experimental settings or the apparently opposing protective or harmful effects of the receptor PAR-1 depending on the activating protease (thrombin or activated protein C) (1–2).

Furthermore, generation of the cytoprotective activated protein C (aPC) depends on thrombin, which is known for its proinflammatory effects and an at least three orders of magnitude better activator of PAR-1 than aPC (3). Obviously, unidirectional signalling pathways are insufficient to model the complexity of coagulation protease-dependent cytoprotective signalling, but rapidly expanding knowledge in the field has yielded new mechanistic insights on which this review will focus.
The carboxyglutamic acid-rich N-terminal domain of aPC binds to EPCR, resulting in an about 10-fold enhanced activation of protein C. However, the initial concept that engagement of EPCR is a prerequisite for the cytoprotective effect of aPC has been challenged. Thus, in human umbilical vein endothelial cells aPC suppresses expression of the pro-apoptotic mediator TRAIL independent of EPCR. Rather, this EPCR-independent function depends on PAR-1 and the co-receptor S1PR1 (sphingosine 1-phosphate receptor-1) (Fig. 2b). These receptors mediate the aPC-dependent repression of TRAIL via ERK-1/2 and EGR-1.

The receptor S1PR1 had been previously shown to mediate aPC-dependent cytoprotective effects, though the exact mode of action remains unclear. Endothelial barrier function, determined via the transendothelial electrical resistance (TER), myosin light chain phosphorylation, cortical actin polymerization, and Rac1 activity were enhanced by aPC via S1PR1. According to Finigan and colleagues aPC may induce a direct interaction between EPCR and the S1PR1, whereby the protective effect of aPC on the endothelial barrier was reduced following silencing of S1PR1 via siRNA (11). Likewise, Feistritzer and Riewald demonstrated a crucial role of S1PR1, in that they suggested a aPC / PAR-1 / EPCR-dependent regulation of the cellular sphingosine kinase-1, increasing S1P generation and subsequent activation of S1PR1 (12).

Thus, while the exact mechanisms proposed by these authors differ, these studies together with the results obtained by O’Brien (13) establish a role of S1PR1 for aPC-mediated cytoprotection in endothelial cells. Of note, in regard to glucose-induced endothelial apoptosis, S1PR1 was found to be dispensable for aPC’s anti-apoptotic function (14). The latter observation is entirely consistent with the protective effects of aPC in a model of experimental diabetic nephropathy in the absence of S1PR1 from renal glomeruli (14–15).

These results indicate that aPC-dependent cytoprotection engages tissue- and cell-specific signaling mechanisms.

Along these lines aPC can signal in monocytic cells (e.g. U937 cells, a human leukaemic monoblast cell line) and in human...
platelets via the apolipoprotein E receptor 2 (ApoER2). Platelets can adhere to immobilized PC or aPC, which induces platelet spreading; this process requires both ApoER2 and GPIb (16). In U937 cells ApoER2 mediates aPC-dependent phosphorylation of Tyr-220 in Dab 1 (adaptor protein diabled-1), of Ser-473 in Akt, and of Ser-9 in GSK3β (glycogen synthase kinase 3β) (17). These reactions are independent of both EPCR and PAR-1, based on antibody inhibition studies. Unfortunately, in vivo data were not available in this report to evaluate whether aPC-dependent signalling independent of PAR1 and EPCR did occur in monocytes and would mediate physiologically relevant processes.

Thus, convincing experimental evidence for a cytoprotective function of aPC independent of its bone fide receptors PAR-1 and EPCR is currently lacking and further investigations are required to solve this open question. Nevertheless, the indicated results imply that aPC-dependent signalling appears to be much more versatile than initially thought, in particular in a cell-specific context.

**Fig. 3**

Thrombin can mediate opposing effects via PAR-1: Low thrombin concentrations (∼50 pmol/l) mediate cytoprotective effects in endothelial cells, while high concentrations (e.g. 100 nmol/l) will activate endothelial cells, resulting in a pro-inflammatory phenotype.

These observations suggest that low levels of thrombin may mediate vasculoprotective effects in vivo.

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**Low dose thrombin and vascular protection**

The versatility of protease-dependent signalling extends beyond activation of specific receptors since, despite engagement of the same receptor a protease can elicit different cellular effects, depending e.g. on its concentrations. Instrumental for our understanding in this regard was the work by Rezaie and colleagues, who identified cytoprotective effects of low dose thrombin. Unlike in most previous studies they explored the effects of thrombin at very low concentrations, ranging as low as 5 pmol/l, whereby 50–75 pmol/l thrombin promoted cytoprotective effects in endothelial cells: Following stimulation with TNF-α, thrombin maintained the endothelial cell barrier function, reduced the expression of adhesion molecules (E-selectin, ICAM-1, VCAM-1), the adhesion of leukocytes, as well as their transmigration through the endothelial cell monolayer (18) (Fig. 3).

Based on inhibitor studies the authors demonstrated that these effects depend on PAR-1 activation and signaling via PI3-kinase (19).

**Tissue protection by prothrombotic risk factors**

Such a protective effect may also underlie the high occurrence of prothrombotic risk factors, such as the prothrombin G20211A mutation or the factor V Leiden (FVL) mutation (R506Q). A positive selection pressure for the FVL mutation was already proposed when this mutation was first described (20). Other mechanisms include reduced peri-puerpal blood loss, improved embryonic implantation, or a reduced lethality in severe sepsis (21–23).

The latter has been a matter of debate, as the initial observed protective effect of the FVL mutation in septic patients or models challenged with lipopolysaccharide (LPS) could not initially be confirmed in clinical follow up studies or in murine sepsis models with bacteraemia. However, when antibiotics were used in the animal models, thus copying more closely the clinical setting, the beneficial effect of the FVL mutation could be recapitulated (24–27). Collectively, these observations indicate that the FVL mutation mediates a protective effect in severe inflammation, but does not reduce the bacterial challenge (e.g. bacterial growth or invasion) per se. The exact mechanism through which FVL mediates these protective effects remains ill-defined. Injection of LPS, which is known to activate thrombin, also enhances PC activation, most likely reflecting the increased interaction between formed thrombin and its receptor thrombomodulin with subsequent PC activation. However, considering the well-established loss of thrombomodulin in septic animals or humans (28–29) other mechanisms underlying the cytoprotective effects of the FVL mutation have to be considered.

New mechanistic insight may be obtained from chronic disease models as well, evaluating a potential protective and beneficial effect of the FVL mutation that may convey large clinical and socio-economic relevance. For example, the presence of the FVL mutation was associated with reduced albuminuria in patients with diabetic nephropathy (30). Likewise, the FVL mutation protected against diabetic nephropathy in a murine model, which was associated with reduced podocyte apoptosis. In diabetic mice, the FVL-mutation correlated with increased plasma levels of thrombin antithrombin but not of aPC, arguing against a protective effect via enhanced PC activation. In agreement with a cytoprotective effect of low but sustained thrombin levels observed in FVL carriers, low thrombin concentrations (50 pmol/l thrombin protected against glucose-in-
duced podocyte apoptosis in vitro whereby hirudin abolished the protective effect observed in diabetic FVL mice (30). In analogy to these protective effects in diabetic nephropathy, a microvascular diabetic complication, slightly enhanced thrombin generation appears to provide protection in macrovascular disease. This conclusion is based on a U-shaped association between F1+2, a marker of in vivo thrombin activation, and recurrent cardiovascular events in the GUSTO study (31). Enhanced thrombin generation has also been related to vasculoprotection in the murine ApoE-/- atherosclerosis model: When receiving a high fat diet, ApoE-/- mice with a superimposed pro-coagulant induction developed larger, but more stable plaques. Again, this effect was lost following anti-coagulation in these mice (32).

This dose-dependency appears not to be specific for thrombin. Unphysiological high concentrations of aPC (>100 nmol/l) may impair the endothelial barrier function, which contrast with the protective function of lower concentrations of aPC (~10 nmol/l) (12). An explanation as to how different concentrations of proteases can provoke such opposing effects remains elusive.

**PAR-signalling**

In addition to the concentration-dependency, the effect of protease-dependent signalling can be determined by the temporal activation pattern. Thus, the function of cell PAR-1 as a cytoprotective or cytodisruptive receptor may depend on the disease stage at which the receptor is activated. In vivo, early inhibition of PAR-1 (at the time when severe inflammation is induced...
Receptor coupling, lipid rafts, ligand promiscuity

The switching of PAR-1 from a protective to a harmful receptor appears to depend on the organization of the receptor complexes within the cell membrane. In endothelial cells the receptors required for cytoprotective signalling are colocalized within lipid rafts, and disruption of lipid rafts by methyl-β-cyclodextrin, a cholesterol-depleting substance may abolish the cytoprotective effect of aPC (34–35). Binding of aPC as well as the zymogen PC to EPCR may trigger dissociation of EPCR from caveolin-1 (Fig. 5a), being associated with a restructuring of receptor complexes. Thus, PAR-1-dependent signalling switches from Gq/11,12 to Gq/12,13 (36). This rearrangement of PAR and companion G-proteins appears to be another example of biased seven-transmembrane receptor signalling (36).

Coupling of PAR to G-proteins is not only regulated by binding of (activated) protein C to EPCR, but also by homo- and heterodimerization of PAR. In endothelial cells, thrombin activation of PAR-1 homodimers results in a preferential activation of Gq/11, while activation of PAR-1 in a preformed heterodimer with PAR-3 results in preferential activation of Gq/13. Hence, the signalling induced and the cellular effects provoked by a specific protease are in part determined by the organisation of the receptor activated in specific membrane domains.

These data established that PAR1 may be switched from a vascular-disruptive to a vascular-protective receptor during the progression of sepsis in mice and identify PAR-2 as a co-receptor required for this switch (33), and illustrate the plasticity of protease-dependent signalling.

These results suggest that the organisation of receptor complexes and their induced rearrangement following binding of cytoprotective ligands are crucial events for the net outcome of cytoprotection or cytodisruption.

The function of EPCR for cytoprotection and rearrangement of receptor complexes are not limited to (activated) PC. EPCR can also bind FVIIa (38), supporting the notion that recruitment and positioning of G-protein-containing proteins other than PC is possible through this co-receptor. Along this line, EPCR promotes PAR-1 cleavage by factor Xa thereby enhancing endothelial barrier function (38), which is consistent with a cytoprotective effect of factor Xa in this specific setting (Fig. 5c). Alternatively, factor Xa can enhance endothelial barrier protection via direct activation of PAR-2 as well (39–40), whereas the activation of PAR-1 by factor Xa appears to depend on EPCR, as outlined above. However, in both cases endothelial protective signalling by factor Xa is associated with a dissociation of EPCR from caveolin-1, activation of Rac1 and inhibition of RhoA (39), resembling the intracellular cytoprotective signalling associated with aPC. Therefore, while the receptor(s) activated by proteases do clearly not determine whether protective or detrimental signalling is initiated, this may nevertheless be determined at the intracellular level by the activation of one common signalling pathway. Future studies evaluating e.g. the role of caveolin-1 or RhoA and Rac1 in other cellular systems will provide additional insights into these process-dependent protective signalling events.

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