Activated protein C based therapeutic strategies in chronic diseases

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Summary
Activated protein C (aPC) is a natural anticoagulant and a potent anti-inflammatory and cytoprotective agent. At the expense of increased bleeding risk aPC has been used – with some success – in sepsis. The design of cytoprotective-selective aPC variants circumvents this limitation of increased bleeding, reviving the interest in aPC as a therapeutic agent. Emerging studies suggest that aPC’s beneficial effects are not restricted to acute illness, but likewise relevant in chronic diseases, such as diabetic nephropathy, neurodegeneration or wound healing. Epigenetic regulation of gene expression, reduction of oxidative stress, and regulation of ROS-dependent transcription factors are potential mechanisms of sustained cytoprotective effects of aPC in chronic diseases. Given the available data it seems questionable whether a unifying mechanism of aPC dependent cytoprotection in acute and chronic diseases exists. In addition, the signalling pathways employed by aPC are tissue and cell specific. The mechanistic insights gained from studies exploring aPC’s effects in various diseases may hence lay ground for tissue and disease specific therapeutic approaches. This review outlines recent investigations into the mechanisms and consequences of long-term modulation of aPC-signalling in models of chronic diseases.

Keywords
Signal transduction, protein C/S pathway, diabetes mellitus, coagulation factors, chronic diseases

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Introduction
Activated protein C (aPC) is a plasma serine protease, providing a feed-back inhibition within the coagulation system. The inactive zymogen protein C (PC) is efficiently activated by thrombin, if thrombin is bound to thrombomodulin (TM). Thrombin bound to TM loses its procoagulant function and acquires activity towards PC activation. The activation of PC by the TM-thrombin complex is about 20-fold augmented by the co-factor EPCR (endothelial protein C receptor) (1). EPCR binds the Gla-domain of PC and facilitates the interaction of PC with the TM-thrombin complex. Activated PC proteolytically inactivates the coagulation co-factors Va and VIIIa, thus providing a negative feed-back on the coagulation system (1, 2).

aPC is not only a major anticoagulant, but in addition is widely recognised as a cytoprotective, anti-inflammatory protease (3-5). The loss of endothelial TM-expression and reduced levels of aPC in patients with poor sepsis outcome (6) rationalised the evaluation of aPC in the context of sepsis. The PROWESS study, the first clinical study evaluating aPC (dotrecogin alfa®) in septic patients, demonstrated a survival benefit (reduced 28-day-mortality) following treatment with aPC during an initial 96-hour period (7). However, the increased bleeding risk remained problematic. Eventually aPC was withdrawn from the market as no survival benefit was evident in subsequent follow-up studies (8, 9). In parallel pre-clinical studies in mice demonstrated that aPC’s cytoprotective effect is independent of aPC’s anticoagulant function in sepsis (10). In a series of elegant in vitro and in vivo experiments different structures of aPC required for its anti-inflammatory effect have been characterised and insights into the receptors and intracellular signalling pathways involved have been obtained (3, 11).

Similar to acute diseases, chronic diseases are associated with reduced levels of aPC or PC. In regard to chronic infectious diseases, enhanced coagulation activation and decreased PC levels have been documented in pulmonary tuberculosis and Helicobacter pylori-associated gastritis (12-14). In a murine model of tuberculosis, impaired PC activation contributes to a hyperinflammatory response (15), but the effect of aPC on murine tuberculosis proved to be only minimal in transgenic mice with constitutively increased aPC levels (16). Enhanced coagulation activation and reduced levels of aPC or PC have been likewise observed in non-infectious chronic diseases, such as type 2 diabetes mellitus, atherosclerosis, myocardial infarction, chronic inflammatory bowel disease, chronic kidney disease or uremia (17-21). In diabetic patients low aPC-levels are associated with vascular complications, such as leg ulcers and carotid atherosclerosis (22, 23). This raised the question as to whether impaired aPC levels are causally involved in the pathogenesis of chronic disease, potentially by modulating the disease progression through altered protease dependent signalling.
The receptor complexes through which coagulation proteases modulate cellular functions are being identified and have been summarised in a recent excellent review (24). The functional separation of aPC’s cytoprotective and anticoagulant properties is an important step towards the adaptation of aPC-based therapeutic approaches in chronic diseases (25, 26). While these and other reviews focused on the effects of coagulation proteases in acute diseases we are at the beginning of understanding the mechanisms underlying aPC’s effect in chronic disease. Of note, as in acute diseases, the cytoprotective effect of aPC appears to be largely independent from its anticoagulant properties in chronic disease.

Cytoprotective aPC analogues

Detailed structure-function analyses established that the anticoagulant and cytoprotective properties are largely mechanistically separable (27, 28). Mutating, for example, positively charged lysine residues required for the interaction with factor Va (KKK191–193) results in aPC-variants with more than 90% reduced anticoagulant but normal cytoprotective function (3K3A-APC) (3, 27). This effect can be enhanced by additional mutations of arginine residues (RR229/230) within the Ca\(^{2+}\)-binding loop, impairing aPC’s anticoagulant activity by more than 98% (5A-APC) (29). Another group introduced single amino-acid substitutes into PC, reducing either aPC’s anticoagulant (K193E) or PAR1-signalling (L8W) activity (30). In a further approach a Cys\(^{67}\)-Cys\(^{82}\) disulfide bond was introduced into PC, stabilising the Ca\(^{2+}\) binding loop and resulting in an aPC mutant with retained cytoprotective, but largely reduced anticoagulant activity (31). Some of these mutant aPC forms with reduced anticoagulant activity have shown equal or even greater efficacy in pre-clinical animal studies in comparison to wild-type aPC (10, 32). More recently, pre-clinical studies evaluating the above aPC variants have been conducted in primates with stroke (33). In addition, a phase 1 study evaluated the 3K3A-APC variant in healthy subjects, demonstrating good tolerability at a concentration of up to 540 µg/kg, while higher doses resulted in moderately severe headache, nausea, and vomiting (34). Although evidence in regard to the safety, cytoprotective efficacy, and mode of action of these aPC-variants is accumulating, further evaluations are necessary, especially if these are considered for long-term use in chronic diseases.

The current knowledge of aPC signalling in chronic diseases is summarized in the subsequent sections. These studies provide promising insight into novel therapeutic approaches of aPC in chronic diseases. Yet the translation of these findings into the clinical practice still constitutes a major challenge. As mentioned above, aPC has an inherent risk of haemorrhage, which precludes an extended use of aPC in humans. While this problem can now be circumvented by using the above-mentioned “signalling-only” variants of aPC, the use of aPC variants as a “biological” will be associated with high costs. Studies evaluating the mechanisms of aPC-dependent cytoprotection in chronic diseases and the design of therapeutic approaches targeting these pathways, preferentially using small molecules, constitute an interesting alternative approach towards feasible therapies mimicking aPC’s cytoprotective effect in chronic diseases.

The role of aPC in diabetic nephropathy

Preclinical studies suggest that the kidney is an organ in which aPC conveys strong cytoprotective effects. Initially, studies focused on acute kidney diseases, such as ischemia reperfusion injury (35) or renal dysfunction associated with systemic infection (30, 36), providing valuable mechanistic insight. Using variant PC analogues (K193E, L8W) or PAR1 antagonists it was shown that PAR1 agonism is required to reverse lipopolysaccharide (LPS)-induced hypotension or renal ischemia-reperfusion injury, respectively (30, 35). Of note, the “signalling only” variant of aPC was sufficient to reduce activation of pro-apoptotic caspase-3 and to abrogate renal dysfunction and pathology (30). Interestingly, in the LPS-model aPC reduced mRNA expression of iNOS, angiotensin-converting enzyme-1 (ACE1), angiotensinogen, while increasing ACE2 (36), demonstrating that aPC modulates pathways involved in chronic kidney injury, such as diabetic nephropathy.

A possible function of aPC in diabetic nephropathy can be deduced from clinical data showing elevated levels of soluble thrombomodulin (TM), which is thought to reflect loss of endothelial TM-function, and reduced levels of aPC in diabetic patients with vascular complications, including nephropathy (23, 37). The evaluation of experimental diabetic nephropathy in mice provided mechanistic evidence for a role of aPC in diabetic nephropathy (38). Genetically impaired PC activation secondary to a targeted point mutation in the TM gene (E404P) (39) aggravates diabetic nephropathy in mice. Contrary, in mice expressing a human PC analogue (D167F/D172K), which can be efficiently activated by thrombin even in the absence of TM, resulting in high levels of aPC, indices of diabetic nephropathy were markedly reduced and not different from non-diabetic controls (38). In agreement with its anti-apoptotic activity aPC protected against podocyte loss and glomerular cell death in genetically modified mice with hyperactivatable PC. In vitro aPC prevented glucose induced mitochondrial apoptosis in glomerular cells at concentrations as low as 2 nM (38). The therapeutic potential of aPC or aPC-based therapeutic strategies was subsequently demonstrated in STZ-injected mice treated intraperitoneally with aPC, which ameliorated parameters of diabetic nephropathy, including albuminuria and podocyte loss (40) (▶Table 1).

Diabetic microvascular complications have been linked with mitochondrial dysfunction and excess reactive oxygen species (ROS) generation, raising the question as to whether aPC’s nephroprotective effect in diabetic mice may be related to mitochondrial function and ROS-generation. Indeed, aPC reduced the glucose induced mitochondrial translocation of the redox-protein p66\(^{scn}\) and mitochondrial ROS-generation at concentrations as low as 2 nM (▶Figure 2) (25). The effect of aPC on p66\(^{scn}\) is apparent in podocytes, but not in glomerular endothelial cells, due to the failure of glucose to induce p66\(^{scn}\) expression specifically in glomerular endothelial cells (25) (▶Table 1). Interestingly, glucose
induces and aPC normalises p66Shc expression in non-glomerular endothelial cells, illustrating the diversity of endothelial cells also in regard to aPC signalling (41). The reduction of ROS by aPC through regulation of p66Shc extends earlier findings, which proposed a direct antioxidant function of aPC in macrophages, albeit at supraphysiological concentrations (42). Yamaji et al. speculated that aPC’s antioxidant effect following LPS stimulation of the macrophage like cell line RAW 264.7 may be related to an inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (42). However, direct evidence for an effect of aPC on NADPH-activity was not presented. Consistent with these in vitro data aPC reduces LPS-induced O2- and nuclear factor (NF)-κB induction in vivo (43). Indeed, these antioxidant activities may be related to the aPC dependent regulation of redox-sensitive transcription factors such as NF-κB and Sp1.

The cytoprotective effect of aPC in podocytes requires PAR3, reminiscent of aPC signalling in neurons (26). However, unlike in neurons, aPC signalling via PAR3 is independent of EPCR, but requires PAR2 (human) or PAR1 (mouse) as a co-receptor (44). Furthermore, in podocytes the cytoprotective effect of aPC was lost following mutation of the “canonical” cleavage site in PAR3 (T39P) (44), contrasting results in endothelial cells, in which a “non-canonical” cleavage site for aPC (Arg41; thrombin cleaves at Lys38) within the N-terminal extracellular domain of PAR3 has recently been identified (45). Unlike in podocytes, cleavage of this non-canonical PAR3 by aPC on endothelial cells requires EPCR (45).

An interaction of PAR3 with PAR1 has been previously shown in endothelial cells, but again mechanisms in endothelial cells and podocytes appear to differ. In endothelial cells PAR3 homo- and heterodimers are pre-formed, whereas aPC induces heterodimerisation of PAR3 in podocytes. At least in podocytes the aPC induced dynamic receptor re-arrangement depends on de-phosphorylation of caveolin-1 (44). These studies illustrate that protease dependent signalling is highly cell- or tissue-specific, requiring detailed studies. This is in agreement with a tissue-specific stoichiometry of the aPC system (44, 46, 47). For example, large arterial vessels are rich in EPCR whereas the capillary endothelial cells have low EPCR levels (48, 49). And, as outlined above, aPC-dependent cytoprotective signalling depends on PAR-1/2/3, but not EPCR in podocytes (44). Hence, the differential effects exerted by aPC in various organs may in part reflect the tissue- and cell-specific receptor pattern and their dynamic (re-) organisation, which have been addressed in excellent recent reviews (24). The delineation of tissue specific mechanism may enable researchers to design targeted therapeutic approaches.

Given the short half-life of aPC the profound effects of aPC-treatment in chronic disease models despite its application once daily or every other day are surprising and indicate the involvement of a “molecular switch” (26, 40, 50, 51) (Table 1). In agreement with this interpretation, the expression of p66Shc is epigenetically controlled by aPC. Intraperitoneal injections of aPC every other day reverse the glucose-induced H3 acetylation and hypomethylation of the p66Shc specific promoter (25). These data establish that aPC epigenetically controls gene-expression (Figure 2), providing a rationale for the long-lasting effect of aPC.

While these studies provided in depth insight into the mechanisms involved in aPC’s nephroprotective effect in the context of diabetes mellitus, a number of questions remain open. Thus, while in vitro and imaging data demonstrated a cytoprotective effect of

Table 1: Application of aPC in chronic disease.

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Mode of aPC application</th>
<th>Outcome of treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic nephropathy</td>
<td>-wt-aPC</td>
<td>-albuminuria ↓</td>
<td>25, 38, 40, 44</td>
</tr>
<tr>
<td></td>
<td>-wt-aPC with blocked anticoagulant function, -i.p. (1 mg/kg, every other day for 4 weeks)</td>
<td>-podocyte dysfunction ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-3K3A-aPC</td>
<td>-ROS ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-i.p., (40–100 μg/kg, daily for 40–80 days)</td>
<td>-p66Shc ↓</td>
<td></td>
</tr>
<tr>
<td>Type-1-diabetes</td>
<td>-wt-aPC</td>
<td>-diabetes incidence in NOD-mice ↓</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>-i.p. (2 mg/kg twice weekly for up to 10 weeks)</td>
<td>-Tregs ↑</td>
<td></td>
</tr>
<tr>
<td>ALS-like disease (mutated SOD1)</td>
<td>-wt-aPC</td>
<td>-survival ↑</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>-3K3A-aPC</td>
<td>-disease progression ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-i.p., (0.2 mg/kg daily for 30 days)</td>
<td>-ROS ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-inhibitory antibody for aPC/PC, -i.p.(1 mg/kg, 4 injections)</td>
<td>-mutant SOD1 ↓</td>
<td></td>
</tr>
<tr>
<td>EAE (experimental autoimmune encephalomyelitis)</td>
<td>-wt-aPC, -L8W-aPC, -K193E-aPC, -i.v. (0.2 mg/kg daily for 30 days)</td>
<td>-exogenous aPC; disease severity ↓</td>
<td>50, 57</td>
</tr>
<tr>
<td></td>
<td>-i.p. (40–100 μg/kg, daily for 40–80 days)</td>
<td>-inhibition of endogenous aPC; MDSCs in the periphery!</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-i.v. (0.2 mg/kg daily for 30 days)</td>
<td>-disease severity ↓</td>
<td></td>
</tr>
<tr>
<td>Diabetic leg ulcers, wound healing</td>
<td>-wt-aPC, -human: topical (0.4 mg/ml saline, twice weekly for 6 weeks)</td>
<td>-wound healing ↑</td>
<td>61, 63–70</td>
</tr>
<tr>
<td></td>
<td>-rats: topical single dose of 20 μg/wound</td>
<td>-angiogenesis ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-inhibitory antibody for aPC/PC, -i.p.(1 mg/kg, 4 injections)</td>
<td>-keratinocyte barrier function ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-3K3A-aPC</td>
<td>-disease progression ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-wt-aPC with blocked anticoagulant function, -i.p. (1 mg/kg, every other day for 4 weeks)</td>
<td>-ROS ↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-wt-aPC</td>
<td>-mutant SOD1 ↓</td>
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activated protein C (aPC) in podocytes and endothelial cells, this has hitherto not been confirmed in vivo, due in part to the lack of suitable mouse models. In addition, it remains currently unknown whether these effects are specific for the kidney, or whether aPC conveys protection from other micro- or even macrovascular complications associated with diabetes mellitus. These studies are ongoing.

Little insight has been obtained into the function of TM`s lectin like domain in chronic disease. In order to act on neurons aPC must cross the blood brain barrier (BBB) via the endothelial protein C receptor (EPCR). In neurons aPC reduces ROS production and nuclear translocation of Sp1 through a PAR1/PAR3 dependent mechanism. The effect of activated PC in experimental autoimmune encephalomyelitis (EAE) is controversial, as exogenous aPC was protective, while endogenous aPC aggravated the disease progression. APC decreases cytokines (IL-17, IL-6, IL-2 and TNF-α) secreted by splenocytes derived from aPC-treated EAE mice. Contrary, blocking endogenous aPC increases the frequency of myeloid derived CD11b+ suppressor cells (MDSCs) and T-cell suppressive factors and decreases CD4+ T-reg-cells in the periphery. In regard to wound healing aPC induces expression of matrix metalloproteinase-2 and VEGF in skin fibroblasts and keratinocytes, supporting a pro-angiogenic effect of aPC in the skin. APC enhances skin integrity and keratinocyte barrier function by activating the tyrosine kinase receptor Tie2, through EPCR, cleavage of PAR1 and transactivation of EGFR.

The role of aPC in neurodegenerative disease

Insights into the signalling mechanisms targeted by aPC can be obtained from studies of aPC`s effect on the central nervous system. In order to act on cells of the central nervous system aPC must cross the blood brain barrier (BBB). EPCR facilitates the transport of aPC and its recombinant variants across the BBB independent of PAR1 (55). Notably, only aPC and its variants, not PC showed high affinity to the BBB-transport (55). This implies a close interaction of the vascular system, which must be functional to ensure PC activation, and the non-endothelial cells within the central nervous system, which are targeted by aPC. The finding...
that aPC’s transport across the BBB depends on EPCR raises the question whether an impaired function of EPCR on brain-endothelial cells may contribute to neurodegenerative diseases.

The potential efficacy of aPC in chronic neurodegenerative diseases has been demonstrated in independent disease models. In a murine model of amyotrophic lateral sclerosis (ALS) aPC and its predominately cytoprotective analogues slow the disease progression and increase survival. This effect is apparent even if treatment is initiated after disease onset (26). APC was intraperitoneally injected for more than 80 days (Table 1), demonstrating the feasibility and efficacy of aPC-based interventions in chronic diseases. In these studies an established mouse model of ALS, which relies on the expression of a mutant superoxide-dismutase 1 (SOD1) and thus reflects the most frequent forms of inherited ALS in humans, was used. Using this disease model, which is based on increased ROS-generation, the authors demonstrated that aPC inhibited neuronal expression of mutated SOD1, ROS generation, and nuclear translocation of the redox-sensitive transcription factor Sp1 (26, 56). Of note, genetically inactivating SOD1 in endothelial cells did not slow the progression of ALS, illustrating a pivotal role of aPC in non-endothelial neuronal cells and the need of aPC to cross the BBB in this neurodegenerative disease model (26) (Table 1).

In neuronal cells, suppression of SOD1 by aPC requires its enzymatic, but not its anticoagulant activity and depends on PAR1 and PAR3 (26) (Figure 1). Once aPC has crossed the BBB it activates PAR3 on neuronal cells through an unknown mechanism. Insight gained from studies in mice with experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis, demonstrated that aPC’s neuroprotective effect in chronic degenerative disease is not restricted to motor neurons, the cell type affected in ALS. Initially, ex vivo studies using biopsies from patients with EAE identified tissue factor and the protein C inhibitor as two main proteins induced in human multiple sclerosis plaques (50). Subsequent in vivo analyses using the MOG-induced mouse model of multiple sclerosis (EAE) demonstrated that exogenous aPC had no impact on the acute disease phase, while ameliorating...
the disease course at later – chronic – stages. Using exogenous application of aPC-analogues the authors concluded that both the anticoagulant and cytoprotective (signalling) activity of aPC is required for neuroprotection in this neurodegenerative disease model (50) (Table 1, Figure 1).

While these studies demonstrate the pharmaceutical potential of aPC based therapies in chronic neurodegenerative diseases, the pathogenic role of endogenous PC-activation (vs exogenous application of aPC) remained to be established. Experimental evidence for distinct functions of endogenous aPC activation vs exogenous therapeutic application came from studies conducted by Alabanza et al. using the same disease model (EAE). Rather than injecting aPC therapeutically they inhibited endogenous aPC using a blocking antibody against PC/aPC (57). This approach, which reduced endogenous levels of aPC, did not worsen, but rather improved the disease course. In detailed analyses the authors demonstrate that blocking endogenous aPC increased leukocyte infiltration in the brain and a subpopulation of CD11b+ cells, known as myeloid derived suppressor cells (MDSCs), in the periphery (57). The increased frequency of leukocytes within the brain is in agreement with an enhanced permeability of the vascular barrier in mice deficient of aPC (57). However, the increased frequency of MDSCs, known to be potent T-cell suppressors, results in an increased expression of T-cell suppressive factors, lower frequency of CD4+ T-cells in the periphery and reduced EAE severity (57) (Table 1, Figure 1). Of note, this effect was only apparent if mice were pretreated with the aPC inhibitory antibodies, but not if PC/aPC was blocked after the disease onset, suggesting specific effects of aPC at various disease stages (57). This is reminiscent of the previous recognised role reversal of PAR1 in murine sepsis models (58) and supports the concept that coagulation protease dependent signalling is not only organ-specific, but in addition organised in a temporal fashion (59). These studies emphasise that a detailed understanding of aPC’s mechanism in cytoprotection is required. Based on these insights clinical studies may fail if the timing of an aPC-based therapeutic approach is wrongly designed.

At the same time the suppression of MDSCs points towards a hitherto unappreciated immunomodulatory function of aPC. MDSCs have an established function in tumour-biology. Indeed, aPC has been shown to hamper tumour growth and metastasis (60). Further studies are required to evaluate whether aPC’s effect on tumour growth are related to MDSCs and whether the time-dependent regulation of aPC during tumour progression is relevant in this context.

The role of aPC in wound healing

The protective potential of aPC has expanded to unforeseen applications. Low circulating levels of the zymogen PC are associated with chronic (> 6 months) lower leg ulcers in diabetic patients independently of gender, type of diabetes, HbA1c-values, or C-reactive protein (22). This raises the question as to whether impaired aPC signalling may be mechanistically linked with impaired wound-healing. Topically applied aPC significantly increased cutaneous wound healing in wild-type and PAR1 knock-out mice, but not in PAR-2 knock-out mice, suggesting PAR-2 to be responsible for cytoprotective – or cytoregenerative – aPC-signalling in this context (61). The exact signalling mechanism of aPC and the relevant cell type targeted by aPC in vivo has not been specified in this context. Interestingly, in mice lacking PAR2 wound-healing is impaired independent of aPC (61), and – at least in vitro – aPC strongly induces PAR2 expression in keratinocytes, suggesting that aPC may not only act by activating PAR2, but alternatively by increasing PAR2 expression (Figure 1). In contrast to the in vivo situation where only PAR2 proved to be relevant, knock down or inhibition of PAR2 or PAR1 reversed aPC induced keratinocyte proliferation and Akt-activation in vitro. However, only inhibition of PAR2 signalling reversed aPC’s effect on p38 phosphorylation in vitro (61). Hence, in wound-healing PAR1 and PAR2 appear to have overlapping, but also distinct functions in this regard.

Signalling of aPC through PAR2 has been reported before (58), but the activation mechanism, in particular in keratinocytes, remains unknown. The tissue factor (TF) factor VIIa complex negatively regulates PAR2 mediated angiogenesis (62). It is possible, but remains to be shown, that enhanced TF-Factor VIIa complex formation and signalling impairs PAR2 dependent angiogenesis in chronic diabetic wounds. Activated PC induces expression of matrix-metalloprotease-2 and VEGF in skin fibroblasts and keratinocytes, supporting a pro-angiogenic effect of aPC in the skin (63). Whether these effects are controlled by TF/VIIa and PAR2 in wound healing remains to be shown. The impact of aPC on angiogenesis makes it an attractive target in chronic wounds regardless (62-64). The question whether signalling via PAR2 is altered in the diabetic state, either by altered receptor expression, altered receptor cofactoring, posttranslational modifications of PARs, or reduced aPC generation is intriguing, but remains to be addressed.

Activation of PAR1 by aPC, which is facilitated by EPCR in keratinocytes, promotes wound healing in vitro (65). In regard to the barrier function of keratinocytes aPC activates Tie2 through a receptor complex comprising EPCR, PAR1 and – via transactivation – the EGFR (66) (Figure 1). EGFR has a crucial function for the development of the epidermis and its appendages. This again supports the concept that differential PARs and co-receptors are recruited in a time- and organ-specific manner to facilitate aPC’s cytoprotective effects.

In agreement with the long-lasting effects of aPC in renal disease, related to posttranslational protein modulation and/or epigenetic gene control (25), a single topical application of aPC is sufficient to promote wound healing in a rat skin healing model (67) (Table 1). During wound healing aPC treatment likewise alters gene expression, increasing for example the expression of VE-cadherin and claudin-1, two junction proteins (66).

These pre-clinical findings led to clinical pilot studies, in which aPC was applied locally to chronic non-improving leg ulcers in patients. In an initial study on four patients topical once-weekly application of aPC over a period of four weeks was well tolerated and improved wound-healing during a four-month follow-up (68). In non-healing wounds following orthopaedic surgery topical treatment with aPC likewise led to a marked improvement, which was
already apparent within one week (69). In a subsequent randomised placebo-controlled pilot trial topical aPC-application twice-weekly for six weeks was evaluated in chronic diabetic leg ulcers (70). Treatment with aPC proved to be safe and increased wound healing as well as the quality of life during the 20-week follow up (70). These studies established that cutaneous long-term application of aPC is well tolerated. In addition, the benefits of cutaneous aPC treatment were apparent well beyond the application period. One may speculate that the benefits of cutaneous, intermittent and short-term aPC application are mechanistically linked to posttranslational modifications and epigenetically controlled gene-expression. Furthermore, aPC’s effect in regard to wound-healing are at least partially related to a direct effect on keratinocytes (66). This corroborates that aPC has effects in non-vascular cells, such as neurons, dermal epithelium, and renal epithelial cells (25, 26, 44, 71).

**Outlook**

The case on aPC in the aftermath of the sepsis trials should not be closed. The evidence discussed here suggests a beneficial role of aPC in chronic diseases, particular in diabetes and its complications. As aPC’s cytoprotective effects are – largely – independent of its anticoagulant activity, cytoprotective-selective non-anticoagulant aPC variants have been generated. The availability of these selective aPC analogues paves the way for new clinical applications of aPC. In addition, current data stress the specificity of aPC dependent signalling in a temporal, spatial and tissue-specific context, indicating that targeted therapies based on this knowledge may become feasible.

The emerging role of aPC in chronic disease poses yet another interesting question. As new anticoagulants are increasingly used in chronic disease the question arises how these will impact aPC-dependent signalling. Indeed, direct thrombin inhibitors have been shown to impair the outcome secondary to the inhibition of aPC activation in an animal model of disseminated intravascular coagulation (72, 73). Furthermore, direct thrombin inhibitors have been linked with an increased cardiovascular mortality in some studies (74). Whether this is related to the inhibition of aPC, or to direct cytoprotective effects of thrombin, remains to be established in future studies.

**Conflicts of interest**

None declared.

**References**


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