Infectious diseases and fibrotic disorders: Potential novel targets
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Protease-Activated Receptors in fibroproliferative disease
Protease-Activated Receptors.

Protease-activated receptors (PARs) belong to the family of G-protein coupled receptors (GPCRs), the largest family of signalling receptors present in the mammalian genome. These typically seven transmembrane domain receptors are activated by a unique proteolytic cleavage mechanism. Currently, four different PARs have been identified, i.e. PAR-1 to -4 (activation and function are excellently reviewed by Borensztajn and Spek). PAR-1, being the prototype of the PAR family, was shown to be involved in thrombin-induced intracellular signaling.\(^2\) Long before the actual identification and characterization of the receptor was a fact, it was shown that thrombin was a potent mitogen for fibroblasts via a direct cellular mechanism which required thrombin's catalytic activity and involved surface receptor activation.\(^3\)\(^-\)\(^5\) Because of the novel and unique activation of the receptor, the characterization of PAR-1 contributed significantly to the understanding of cellular activation, not only by thrombin but by proteases in general.\(^2\) Not long after the identification of PAR-1, three family members were identified. First, PAR-2 was identified by a homology search using a murine genomic library screen.\(^6\) Interestingly, PAR-2 was activated by low concentrations of trypsin and by a peptide (SLIGRL) derived from the receptor sequence, but was not activated by thrombin. The subsequent generation of PAR-1 deficient animals ultimately led to the identification of the other two known PARs. As expected, cells derived from these PAR-1 knockout mice could not be activated by thrombin, but platelets derived from the same animals could still be activated very efficiently by thrombin.\(^7\) These data suggested that (at least on platelets) alternative thrombin receptors were present. Indeed, a PCR-based strategy led to the identification of PAR-3 as a novel member of the thrombin receptor family.\(^8\) Interestingly however, thrombin responses were delayed in platelets from PAR-3 deficient mice but they were not absent suggesting the presence of yet another PAR family member.\(^9\) In fact, a GenBank BLAST search for PAR-related sequences led to the identification of PAR-4\(^10\) as a protein with over 30% amino acid identity with PAR-3. Subsequent studies showed that PAR-1 and PAR-4 mediate thrombin signalling in human platelets, whereas PAR-3 and PAR4 are the functional thrombin receptors in murine platelets.\(^11\) As already mentioned above, PARs belong to the superfamily of GPCRs and therefore display several structural features representative of this family, including the seven transmembrane domains. Sequence alignment of PARs with rhodopsin, the prototype GPCR receptor, reveals that indeed the transmembrane domains are highly conserved between rhodopsin and the individual PARs.\(^1\) Moreover, the four PAR family members itself are highly homologous with respect to their amino acid sequence (as expected)\(^1\).
As mentioned before, PARs are activated by proteolytic cleavage of the N-terminal part of the receptor thereby exposing a new tethered ligand (Figure 1A). The new N-terminal extracellular part of the receptor subsequently folds back and interacts with the second extracellular loop of the receptor thereby inducing transmembrane signaling to G proteins (Figure 1B). Due to the autocatalytic activation, a six amino acid long peptide sequence (SFLLRN for PAR-1 and SLIGKV for PAR-2) derived from the N terminus of the individual PARs is able to activate the receptor in the absence of protease-mediated cleavage of the N terminus (figure 1C). Notably, PAR-3 behaves a bit different from the other family members as cleavage does not seem to induce cellular signal by itself. Instead, murine PAR-3 acts as a cofactor for PAR-4 by binding and localizing thrombin for activation of PAR-4. PAR-3 thereby enhances the efficacy of PAR-4 as this receptor has a low affinity for thrombin itself. Whether PAR-3 exerts a similar role in humans or can signal by itself remains to be established.

Once activated, PARs produce a short-lived signal due to rapid inactivation. Several signaling pathways induced by PAR activation have been described over the years (see reference 11 and 12 for excellent review). Most important are Ca\(^{2+}\) mobilization, the MAP kinase/Erk1/2 pathway, the JNK1/2 pathway and the Protein Kinase C pathway. Detailed description of the different signaling pathways goes beyond the scope of this introductory chapter and for more details see the reviews mentioned above.

The recent discovery of novel cell-penetrating peptides (called pepducins) that are able to block GPCR signal transduction, thereby acting as intracellular inhibitors of the receptor, is of great interest. Pepducins are highly specific to a certain receptor and are able to abolish the induction of signal transduction upon activation of the receptor. The pepducins are designed such that they will bind to the third intracellular loop of the receptor and upon interaction they prevent binding of the receptors cognate intracellular signal transducer proteins (Figure 1D). The utilization of pepducins provides a simple and powerful tool to determine the pharmacological disruption of PAR signaling and will be useful to validate and extent knowledge on previously obtained results obtained using genetic ablation of PARs. For PAR-1, 2 and 4 specific pepducins have been described, whereas for PAR-3 the development of a specific pepducin is not required as the receptor does not signal by itself. Several other mechanisms blocking PARs have also been described, (excellently reviewed by Ramachandran et al.), such as extracellular blocking molecules, but are beyond the scope of this introductory chapter.
In most GPCRs, receptor signaling is terminated by dissociation of the agonist from the receptor, but PARs are not inactivated after ligand dissociation. In general for GPCRs, β-arrestin binding to the intracellular domain of the receptor promotes rapid uncoupling from G protein after which the uncoupled GPCRs are internalized and recycled to the membrane. Like GPCRs, activated PARs are rapidly uncoupled by arrestin binding. Unlike GPCRs however, the proteolytic cleavage of PARs render their activation irreversible and are therefore not recycled to the membrane, but internalized PARs are sorted directly to lysosomes for degradation, thereby preventing PARs from returning to the cell surface for continues signaling. Consequently, responses to PAR agonists are rapidly attenuated and strongly desensitized to repeated exposure. This general mechanism holds true.

**Figure 1**: Schematic representation of PAR activation. Proteolytic cleavage of the N-terminal domain reveals a new tethered ligand (A) that folds back onto the core of the receptor thereby activating the receptor signalling pathways (B). The agonist peptide (peptide sequence identical to the tethered ligand) activates the receptor in the absence of activating proteases (C). Specific pepducins bind to the intracellular part of the receptor thereby blocking pathway activation (D).
for PAR-1, -2 and -4. Whether cleaved PAR-3, which as indicated above, does not interact with G proteins, is also internalized after cleavage remains to be established. Next to this general mechanism for PAR inactivation, alternative mechanisms accounting for termination of PAR signalling have been described. For instance, PAR-1 signaling is controlled by constitutive endocytosis\textsuperscript{23}, but whether such a mechanism holds true for other PARs has not yet been determined. Interestingly, proteolytic cleavage downstream of the tethered ligand can lead to de-activation of the receptor as the tethered ligand will be lost.\textsuperscript{24} This might be a potentially important mechanism in the regulation of PAR signaling and one could argue that the effect of PAR agonists might be dampened due to prior “disarming” of the receptor.

**Protease-Activated Receptors and fibroproliferative disease.**

In the setting of lung injury and pulmonary fibrosis, recent data suggest that PAR-1 and PAR-2 both induce pro-inflammatory and pro-fibrotic processes that aggravate disease progression. Activation of PAR-1 enhances inflammation in the pulmonary epithelium, it induces the (myo)fibroblasts differentiation and it potentiates extracellular matrix synthesis.\textsuperscript{25-27} In addition, the absence of PAR-1\textsuperscript{28} limits bleomycin-induced acute lung inflammation and fibrosis, as evident from decreased levels of proinflammatory and profibrotic mediators like transforming growth factor (TGF)-β, interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1) in combination with reduced total collagen levels in the lung. Moreover, PAR-1 expression is increased within fibroproliferative and inflammatory foci in patients suffering from idiopathic pulmonary fibrosis (IPF).\textsuperscript{26}

The activation of PAR-2 induces acute lung inflammation and triggers fibroproliferative responses like fibroblast proliferation, migration and differentiation into myofibroblasts.\textsuperscript{29-31} Importantly, genetic ablation of PAR-2 provides protection from bleomycin-induced pulmonary fibrosis, as evident from a reduction in the extent and severity of fibrotic lesions and diminished collagen expression.\textsuperscript{32} Finally, PAR-2 expression is increased in lungs of IPF patients and PAR-2 expression highly correlates with the extent of honeycombing.\textsuperscript{32-34}

The role of PAR-1 and PAR-2 in fibrotic disease is not limited to the pulmonary compartment. Indeed, PAR-1 deficiency limits liver fibrosis in experimental liver fibrosis as evident from a reduced area of activated fibrogenic cells in PAR-1 heterozygous and PAR-1 deficient mice as compared to wildtype controls and reduced collagen type I, matrix metalloproteinase-2 and Platelet-derived growth factor receptor mRNA levels.\textsuperscript{35} Moreover, PAR-2
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is also implicated in driving fibrotic disease in different organs like liver\textsuperscript{36} and kidney\textsuperscript{37} by modifying fibroblast proliferation and/or ECM deposition. As for the lung, several studies show that PAR-1 and PAR-2 are highly expressed during fibrotic disease. Of importance, in the skin, PAR-1 is expressed on keratinocytes, endothelial cells and fibroblasts and the density of PAR-1 positive fibroblasts is increased in the skin of SSc patients compared to that of healthy controls.\textsuperscript{38} Moreover, PAR-1 is abundantly expressed in both the epidermis and dermis of normal and hypertrophic scars (a common complication following burn wounds) and in keloid lesions.\textsuperscript{39} Next, PAR-2 is expressed in myofibroblast rich regions in keloid and hypertrophic scars\textsuperscript{39}, whereas PAR-2 expression is increased in dermal fibroblasts of scleroderma patients.\textsuperscript{38} The relevance of PAR-1 and PAR-2 expression in the skin for the development/progression of (burn)wound-induced skin fibrosis is part of the work described in this thesis (Chapters 10 and 11).

Overall, the current knowledge highlights PAR-1 and PAR-2 as critical contributors in promoting fibroproliferative disease. Whether PAR-3 and PAR-4 also play an important role in the development of fibrosis \textit{in vivo} remains elusive.
REFERENCES
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