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Protease-Activated Receptors: A Means of Converting Extracellular Proteolysis into Intracellular Signals

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It was known for many years that proteases such as thrombin and trypsin were capable of exerting specific hormone-like effects on cells. Such effects included mitogenesis and platelet activation, and were known to be dependent on the proteolytic activity of the enzymes. The search for receptors for thrombin was frustrated by the inability of many researchers to show binding of the enzyme with an appropriate affinity to cells in classical radioligand-binding assays. An explanation for these problems came with the description in 1991 of the first of the protease-activated receptors (PARs), a thrombin receptor now known as protease-activated receptor-1 (PAR-1; ref. 1). It became clear that members of this new group of receptors, although belonging to the seven-transmembrane domain G protein-coupled receptor family, had a mechanism of activation quite different from that of known receptors. The protease cleaves the extracellular domain, creating a new N-terminus that acts as a 'tethered ligand', which in turn binds to one of the extracellular loops of the receptor and elicits intracellular signalling events (**Fig. 1**; ref. 1). Thus, the

role of the protease is to change the structure of the receptor in such a way that it becomes its own activator. The failure of earlier workers to detect high affinity binding of thrombin to cells could be explained by the fact that only transient binding of the enzyme to the extracellular domain of the receptor is required for activation to occur. The use of homology cloning rapidly led to the identification of PAR-2 as a trypsin receptor (2). The demonstration that cells from PAR-1-null mice could still respond to thrombin led to a search for further thrombin receptors, and the identification of PAR-3 and PAR-4 ensued (3, 4).

Receptor activation and desensitisation

The nature of the mechanism of activation of PARs has implications for many aspects of the biological function of these receptors, which differentiate them fundamentally from other G protein-coupled receptors.

Some of these differences involve the mode of activation itself. The transient nature of the interaction of enzyme with receptor suggests that one protease molecule may conceivably activate several PAR molecules. In addition, any protease

capable of cleaving the N-terminal domain of a PAR exclusively at the site of activation is capable of activating it. This fact does not mean that the PARs are particularly promiscuous. Indeed, PAR-1 and PAR-3 appear to be relatively specific for thrombin as far as mammalian proteases are concerned. Trypsin can activate PAR-1, but at much higher concentrations than thrombin (1). The selectivity of thrombin for PAR-1 and PAR-3 appears to be conferred by the presence in their N-terminal extracellular domains of a sequence analogous to that of a thrombin-binding region in the leech-derived thrombin inhibitor, hirudin (4). PAR-4, which does not possess the hirudin-like sequence, is activated by cathepsin G as well as by thrombin (4). PAR-2, which is not activated by thrombin, appears to have a broader range of cognate proteases; in addition to trypsin, mammalian proteases shown to activate PAR-2 include mast cell tryptase, the cell membrane-anchored membrane-type serine protease 1 and the sperm protease acrosin (5, 6).

Under experimental conditions, PAR-1, PAR-2 and PAR-4 can be activated by synthetic peptides corresponding to the N-terminal tethered ligand sequence in

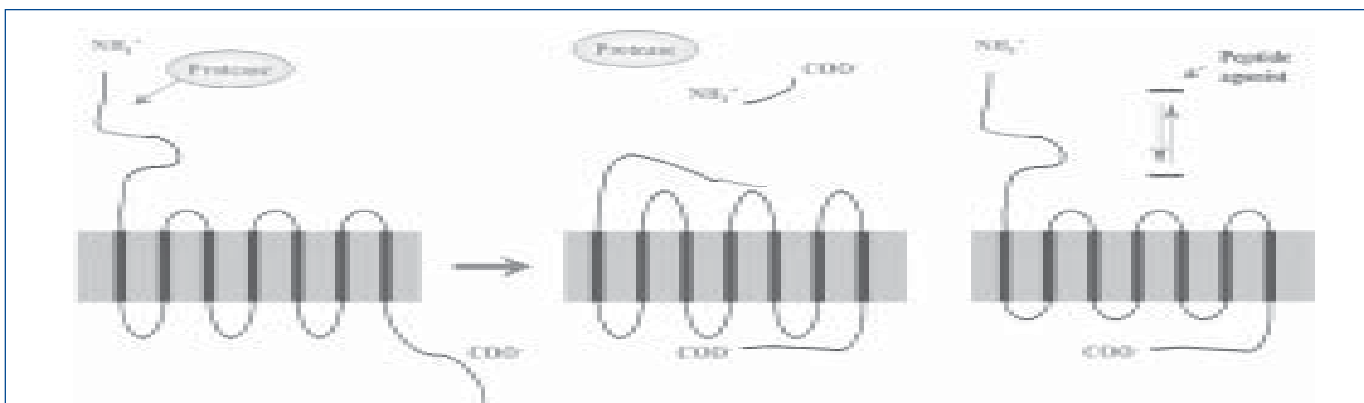


Figure 1: Mechanism of activation of protease-activated receptors. The left and centre panels depict the activation of a PAR by proteolytic cleavage of the N-terminal extracellular domain. This leads to creation of a new N-terminus which acts as a tethered ligand, binding to the second extracellular loop of the receptor and initiating intracellular signalling. The right panel depicts the activation of a PAR receptor by a peptide consisting of 6 amino acids in a sequence identical to the new N-terminus which is exposed by proteolytic activation.

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the absence of proteolysis (5) (Fig. 1). These receptor-activating peptides are very useful tools, allowing the specific experimental activation of PARs in cultured cells or whole animals, without the more general detrimental effects that may accompany treatment with active proteases. Pharmaceutical compounds based on the tethered ligand sequences are now being developed as both agonists and antagonists of protease-activated receptors.

Another aspect of PAR function influenced by the unique mechanism of activation is receptor lifespan. Clearly, once a PAR has been cleaved and activated, it cannot be activated again by a subsequent administration of protease. The events that follow activation have been best characterised for PAR-1 (4). Following activation of PAR-1 by thrombin, signalling is terminated and the receptor internalised by phosphorylation-dependent mechanisms. Rather than being recycled to the cell surface like other G protein-coupled receptors, PAR-1 molecules are then delivered to lysosomes for degradation. The cell remains refractory to further thrombin treatment until new PAR-1 molecules (freshly synthesised or already present in intracellular stores) are delivered to the cell surface.

Biological roles of protease-activated receptors

Protease-activated receptors mediate a variety of cellular responses in different tissues. Since it is not possible to cover all aspects of PAR function in this short review, a selection of the responses elicited by activation of the thrombin receptors and PAR-2 will be discussed.

Thrombin receptors

Thrombin is generated from prothrombin in blood and has a critical role in blood coagulation; it is not surprising, therefore, that much of the available information about thrombin receptors relates to vascular biology. Thrombin contributes to blood coagulation not only through its cleavage of fibrinogen to form fibrin, but also through platelet activation, which

comprises a complex set of responses including shape change, aggregation, and secretion of vasoactive substances and growth factors. Protease-activated receptors mediate thrombin-induced platelet activation, although the identity of the PARs involved differs between species (4). Human platelets express PAR-1 as a high affinity thrombin receptor and PAR-4 as a low affinity receptor. In contrast, mouse platelets lack PAR-1, but instead express PAR-3 and PAR-4. This is not, however, a simple equivalent of the two-receptor system seen in human platelets, since mouse PAR-3 expressed in heterologous expression systems is unable to mediate signalling responses to thrombin. Mouse PAR-3 appears to be required as a cofactor for optimal activation of mouse PAR-4 at low concentrations, probably assisting in binding thrombin to the platelet surface and presenting it to PAR-4 (4). In contrast, PAR-4 is absolutely required for mouse platelet activation, as demonstrated in studies using platelets from PAR-4-null mice (7).

Not only does thrombin exert effects on circulating blood cells, but also on cells of the vessel wall. Vascular endothelial cells and smooth muscle cells express PAR-1, which is now known to mediate many of the responses of these cells to thrombin. Thrombin affects endothelial cells in ways that would be expected to contribute to both thrombosis and inflammation; for example, thrombin stimulates endothelial release of von Willebrand factor, and increased permeability of endothelial monolayers (4). PAR-1 mediates thrombin-induced proliferation in vascular smooth muscle cells (5). Confirmation of the importance of PAR-1 in the vasculature is starting to emerge from studies in PAR-1-null mice. Two groups have independently produced such mice, and both have described death of approximately half of the homozygous PAR-1-null embryos at mid-gestation, with bleeding at multiple sites (3, 8). This embryonic loss can be reversed by targeted expression of PAR-1 in endothelial cells, indicating that responses to thrombin by endothelial cells are important in vascular development in the

mouse (9). The use of PAR-1-null mice has also led to demonstration of roles for PAR-1 in microvascular permeability and in extracellular matrix deposition during the response to vascular injury (8, 10).

Receptors for thrombin are also expressed by non-vascular cells in many tissues. In such tissues, the thrombin necessary for receptor activation may be generated from locally expressed prothrombin, or may be present as a result of loss of vascular integrity through injury or inflammation. Examples of tissues where prothrombin and PAR-1 are known to be co-expressed are the brain and skeletal muscle. PAR-1-mediated actions of thrombin in cells derived from nervous tissue include neurite retraction and astrocyte proliferation (5, 11). PAR-1-mediated thrombin-induced proliferation of skeletal myoblasts, which has been demonstrated *in vitro*, is likely to contribute to early neonatal muscle development where simultaneous expression of prothrombin and PAR-1 occurs (12, 13). In bone, PAR-1 mediates thrombin-induced osteoblast proliferation; PAR-1 expression in osteoblasts is induced by transforming growth factor- β , and is likely to contribute to the responses of osteoblasts to this potent stimulator of bone formation (14).

Protease-activated receptor-2

The first enzyme shown to activate PAR-2 was trypsin (2). Because this enzyme is present in the gastrointestinal tract, where PAR-2 is known to be expressed, much of the early attention was focussed on analysing the role of the receptor in this organ system. PAR-2 activation leads to gastric smooth muscle contraction and prostaglandin release by intestinal epithelium (15). The identification of mast cell tryptase and subsequently other proteases as activators of PAR-2, together with distribution studies demonstrating PAR-2 expression in a wide range of developing and adult tissues, considerably broadened the range of tissues in which roles for PAR-2 were sought (5, 16).

In the vasculature, PAR-2 mediates proliferative responses of endothelial and

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smooth muscle cells. Activation of PAR-2 leads to endothelium-dependent vasodilation of isolated vascular preparations, and a lowering of blood pressure in rats (5). Responses in many tissues indicate a role for PAR-2 in potentiation of inflammatory responses. For example, activation of PAR-2 on keratinocytes and oral epithelial cells leads to release of inflammatory mediators such as interleukin-6 (5, 17), and the onset of inflammation is delayed in PAR-2-null mice due to a defect in leukocyte rolling (18). On the other hand, PAR-2 activation in airways causes a rapid prostaglandin-mediated epithelium-dependent airway relaxation, which helps to alleviate the detrimental effects of airway inflammation (19).

Another interesting action of PAR-2, with implications for the development of therapeutic compounds, is the promotion of skin pigmentation by stimulating keratinocytes to phagocytose the pigment-producing melanocytes. These observations have led to the suggestion that protease inhibitors could be used as depigmenting compounds to treat patients with disorders involving excessive pigmentation (5).

Activation of PARs by bacterial proteases

Many pathogenic bacteria produce proteolytic enzymes that contribute to their pathogenicity by directly causing tissue destruction. The possibility that some of these proteases may also contribute to pathology through activation of host cell PARs has recently been proposed. The bacterium *Porphyromonas gingivalis* is known to contribute to the pathogenesis of periodontitis, a disease of the tooth-supporting tissues which not only leads to tooth loss, but has also been associated with heart disease. Two related cysteine proteases known as gingipains-R produced by *P. gingivalis* have been shown to activate between them PAR-1, PAR-2 and PAR-4. There is evidence that these enzymes contribute to the pathophysiology of periodontitis by acting on PARs expressed by a variety of cell types (17, 20). Of particular interest is

the demonstration that gingipains-R are potent stimulators of platelet aggregation, providing a mechanism for the link between periodontitis and cardiovascular disease (20).

Intracellular signalling elicited by protease-activated receptors

Protease-activated receptor-1 is the best studied of the PARs with respect to intracellular signalling (4). PAR-1 can couple to members of the $G_{12/13}$, G_q and G_i families of G proteins, resulting in activation of multiple pathways. Rho-dependent cytoskeletal responses are likely to mediate responses involving shape change such as platelet activation and endothelial permeability. Activation of phospholipase C_β and subsequent phosphoinositide hydrolysis lead to calcium mobilisation and activation of protein kinase C. Additional responses include inhibition of adenylate cyclase and activation of phosphoinositide 3-kinase. Downstream responses to these pathways include activation of a plethora of kinases such as mitogen-activated kinases, calcium-regulated kinases, and Rho-activated kinase. The breadth of these responses mediated by a single member of the PAR family help to explain the diversity of thrombin's cellular actions.

Future research

Protease-activated receptors are the subject of a rapidly expanding area of research. Four PARs have been described since the publication of Vu *et al.* (1) ten years ago, and it cannot be predicted whether there are more to be discovered. There is also still much to be learnt about the four known PARs. In many cases, PARs are expressed in tissues where expression of known activators has not yet been described. Are the 'thrombin receptors' in many non-vascular tissues just called into action when there is perturbation of vascular integrity, or are there as yet undiscovered local activators that allow these receptors to participate in normal cellular metabolism? For some of the PARs, expression but not function has been identified in certain tissues. Many questions remain unanswered

concerning mechanisms of activation, interactions between PARs and signalling mechanisms, ensuring that this will be an active area of research for many years to come.

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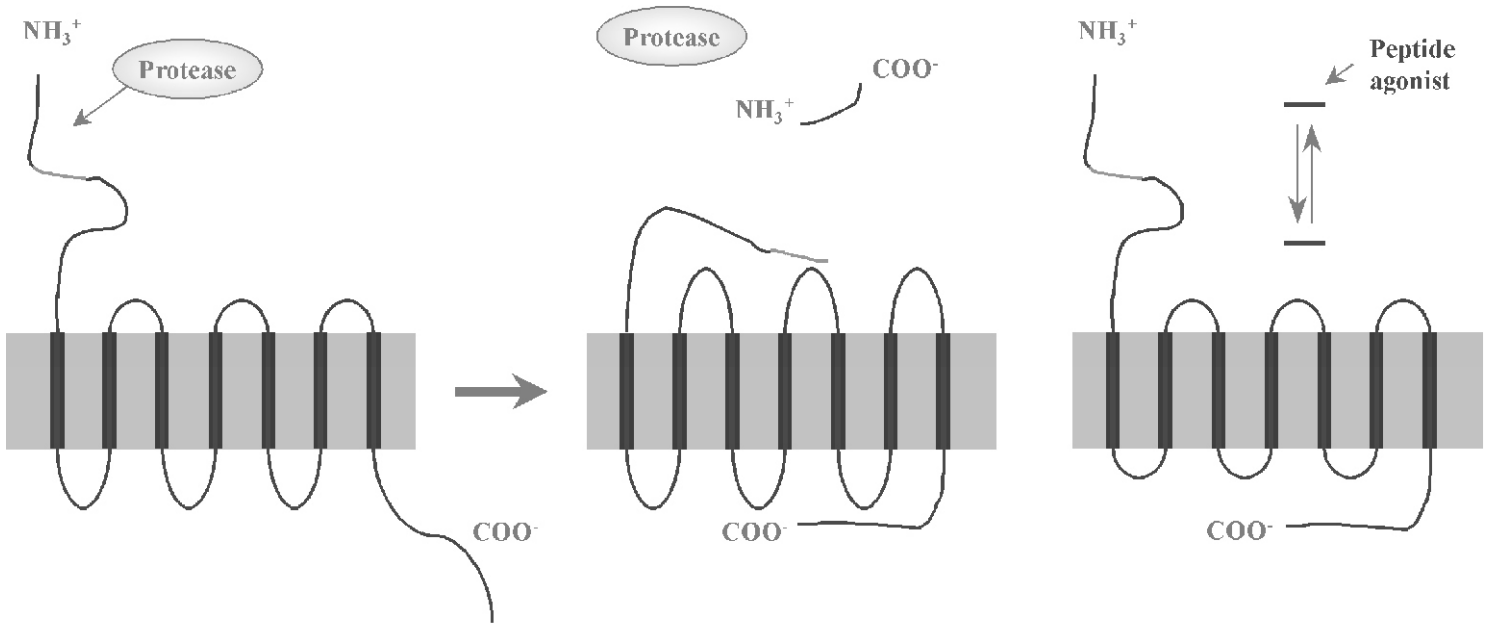


Fig. 1