

## Research Overview

## The Inflammatory Response

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

**ABSTRACT** Inflammation, which constitutes the body's response to injury, is characterized by a series of events where several different cell types are playing distinct roles. Proteinase-activated receptors (PARs) are expressed in all the cell types that are involved in inflammatory processes. The expression of PARs is up-regulated during inflammatory processes. Activation of PARs can lead to inflammation or, in some circumstances, can be protective against uncontrolled inflammatory reaction. Most recently, studies have shown that inflammatory responses were altered in PAR-deficient mice. Thus, a crucial role for PARs seems to emerge from the most recent literature and presents PARs as novel and interesting therapeutic targets for the treatment of inflammation. *Drug Dev. Res.* 59:375–381, 2003. © 2003 Wiley-Liss, Inc.

**Key words:** proteinases; proteinase-activated receptors; thrombin; trypsin; tryptase; inflammation; leukocyte

## THE INFLAMMATORY REACTION

Inflammation constitutes the body's response to injury and is characterized by a series of events that includes the *inflammatory reaction per se*, a *sensory response* perceived as pain, and a *repair process*. The inflammatory reaction is characterized by successive phases: (1) a silent phase, where cells resident in the damaged tissue release the first inflammatory mediators, (2) a vascular phase where vasodilation and increased vascular permeability occur, and (3) a cellular phase, which is characterized by the infiltration of leukocytes to the site of injury. The sensory response includes pain, hyperalgesia, which is defined as an exaggerated response to a noxious stimulus, and allodynia, which is defined as a nociceptive response to a normally innocuous stimulus. The repair process includes tissue cell division, neovascularization and re-innervation of repaired tissues. In many diseases such as arthritis, inflammatory bowel disease, and asthma, the inflammatory process is not appropriately regulated. As a result, significant tissue dysfunction (leading to the generation of the symptoms that typify these diseases), and tissue re-structuring occur (e.g., fibrosis) that can further impair tissue function. Among the many mediators that are involved in the coordination of inflammatory responses are a group of enzymes known

as proteinases. The principal function of proteinases has traditionally been considered to be protein degradation. However, compelling evidence indicates that certain proteinases, notably thrombin, trypsin, mast cell tryptase, and cathepsin G, also function as signaling molecules that regulate target cells by activating members of a newly identified family of receptors: the proteinase-activated receptors (PARs). Here, we summarize evidence that PARs are not only expressed by all the cells that are actively involved in the inflammatory processes, but also that PAR activation affects all aspects of inflammation: the inflammatory reaction, the sensory response, and the repair process.

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Abbreviations used: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; NK-1, neurokinin-1; PARs, proteinase-activated receptors; PAR-AP, proteinase-activated receptor-activating peptide.

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### EXPRESSION OF PARs IN CELLS INVOLVED IN INFLAMMATION

Although originally described in platelets and endothelial cells [Dery et al., 1998], PARs have also been detected in many other cell types, particularly in cells involved in inflammatory processes. PAR<sub>1</sub> has been detected in fibroblasts, monocytes, T cells, natural killer cells, hematopoietic progenitor cells, smooth muscle cells, epithelial cells, neurons, glial cells, and mast cells [Macfarlane et al., 2001]. PAR<sub>2</sub> is expressed by various epithelia (lung, gastrointestinal tract, for instance), as well as endothelial cells, smooth muscle cells, fibroblasts, nerves, and various immune and inflammatory cells, such as T cells, monocytes, macrophages, neutrophils, mast cells, or eosinophils [Macfarlane et al., 2001]. PAR<sub>3</sub> expression has been described in numerous tissues, such as bone marrow, heart, brain, placenta, liver, pancreas, thymus, small intestine, stomach, lymph nodes, and trachea, but the exact cell types that express PAR<sub>3</sub> in those tissues remain to be identified. In rodents, PAR<sub>3</sub> is expressed in platelets and megakaryocytes [Macfarlane et al., 2001]. Although PAR<sub>3</sub> is considered as a co-factor for PAR<sub>4</sub> activation by thrombin, PAR<sub>3</sub> is also found in several tissues where PAR<sub>4</sub> is not present, and vice versa, PAR<sub>4</sub> is present in some tissues independent of PAR<sub>3</sub>. This suggests that in some tissues, PAR<sub>3</sub> might not act only as a co-factor, but can also generate intracellular signals, and that PAR<sub>4</sub> might not need PAR<sub>3</sub> to be activated, thus giving more strength to the hypothesis that in some cell types, PAR<sub>4</sub> might be activated by proteinases different from thrombin. Except for platelets and airway epithelial cells, where PAR<sub>4</sub> expression has clearly been defined, the exact cell types that express PAR<sub>4</sub> in brain, liver, pancreas, or gastrointestinal tract, still have to be defined.

The fact that PARs are expressed by all the cellular actors of inflammation, together with the upregulation of PAR<sub>2</sub> expression in endothelial cells in response to inflammatory mediators such as interleukin (IL)-1, TNF $\alpha$ , or even bacterial lipopolysaccharide (LPS) [Cicala et al., 1999b; Nystedt et al., 1996], strongly suggests a role for PARs, and more particularly for PAR<sub>2</sub>, in inflammatory processes.

### ACTIVATION OF PARs LEADS TO INFLAMMATION

Activation of PARs affects all the aspects of inflammation: the inflammatory reaction per se, the sensory response, and the repair process. Although platelet activation constitutes an important event of the inflammatory reaction, we did not mention the interactions of PARs with platelets in this article (for this particular aspect, see Perini and Wallace, this

Special Issue of Drug Development Research 59(4):395).

### INFLAMMATORY REACTION

The very first event of the inflammatory reaction, the "silent phase," is based upon the reaction of resident cells of the damaged tissue. Among these resident cells, mast cells and macrophages are playing a determinant role in alerting the body to tissue injury, by releasing mediators, such as nitric oxide (NO), histamine, kinins, cytokines, or prostaglandins. PAR<sub>1</sub> and PAR<sub>2</sub> are expressed on mast cells, where their activation leads to mast cell activation and subsequent release of mast cell granule contents [D'Andrea et al., 2000]. PAR<sub>1</sub> and PAR<sub>2</sub> expression has also been described on monocytes and macrophages, where their activation leads to the release of inflammatory cytokines such as interleukin (IL)-1, -6, and -8 [Naldini et al., 1998]. Epithelial cells can also release cytokines (IL-6, IL-8), and prostanoids in response to PAR<sub>1</sub> or PAR<sub>2</sub> agonists [Asokanathan et al., 2002; Kong et al., 1997]. These studies suggest that PAR<sub>1</sub> and PAR<sub>2</sub> activation on resident mast cells, macrophages, or epithelial surfaces, could participate in the surveillance of tissue integrity, and could take an active part in the initiation of inflammatory processes in response to tissue injury.

The release of vasomotor mediators from resident cells leads to the second phase of the inflammatory reaction: the vascular phase. Here again, PARs seem to play a prominent role in the regulation of vascular permeability and motor functions associated with inflammatory response. In vivo, intravenous administration of PAR<sub>2</sub> agonists caused severe hypotension [Cicala et al. 1999a]. It has been shown that both PAR<sub>1</sub> and PAR<sub>2</sub> regulate vascular tone by an endothelial-dependent mechanism involving the release of nitric oxide [Al Ani et al., 1995; Hamilton and Cocks, 2000; Hollenberg and Compton, 2002; Roy et al., 1998] (see Cirino, this Special Issue of Drug Development Research 60(1)). PAR<sub>1</sub> agonists have been shown to induce prostanoid generation and increased barrier permeability in cultured endothelial cells [Vogel et al., 2000]. Both PAR<sub>1</sub> and PAR<sub>2</sub> agonists induced the release of pro-inflammatory cytokines such as IL-6, IL-8, TNF $\alpha$ , by cultured endothelial cells [Chi et al., 2001; Naldini et al., 2000]. In vivo, PAR<sub>1</sub> and PAR<sub>2</sub> agonists caused increased vascular permeability [Cirino et al. 1996; Vergnolle et al. 1999a,b; de Garavilla et al. 2001; Steinhoff et al. 2000]. The intraplantar injection of PAR<sub>1</sub> and PAR<sub>2</sub> agonists in rats led to a severe edema, which lasted for several hours [Cirino et al., 1996; Vergnolle et al., 1999a,b]. Further studies have shown that PAR<sub>1</sub> and PAR<sub>2</sub> agonist-induced edema was

largely mediated by a neurogenic mechanism, where neurokinin-1 (NK-1) and calcitonin gene-related peptide (CGRP) receptors are involved [de Garavilla et al., 2001; Steinhoff et al., 2000]. These studies suggest that PAR<sub>1</sub> and PAR<sub>2</sub> agonists signal to sensory afferents, inducing the release of neuropeptides (substance P and CGRP), which are known to act on vascular beds to induce vasodilatation and increased permeability. As a result, these two vascular events provoke plasma leakage from the blood to the inflamed tissues, and facilitate the passage of leukocytes from the blood flow to the tissues, initiating the third phase of the inflammatory reaction: the cellular phase.

The cellular phase of the inflammatory reaction is characterized by the arrival to the site of inflammation of leukocytes circulating in the blood. In order to be recruited to the site of inflammation, circulating leukocytes have to start rolling onto the venular endothelial surfaces; they then have to adhere to the endothelium, in order to transmigrate across the endothelial barrier. All those events of rolling, adhesion, and transmigration are regulated by several adhesion molecules expressed both by the endothelium and the leukocytes. Thrombin is known to induce rolling, adhesion, and transmigration of leukocytes across the endothelium [Vergnolle et al., 2002]. Although PAR<sub>1</sub> is expressed both on the endothelium and leukocytes, in a recent study, we have shown that these thrombin-induced rolling and adhesion events were not inhibited by a treatment with a PAR<sub>1</sub> antagonist [Vergnolle et al., 2002]. This suggests that thrombin-induced leukocyte recruitment is not mediated by the activation of PAR<sub>1</sub>. However, selective PAR<sub>4</sub>-activating peptides were able to reproduce the effects of thrombin on leukocyte rolling and adhesion, suggesting a potential role for PAR<sub>4</sub> in thrombin-induced leukocyte recruitment [Vergnolle et al., 2002]. Selective PAR<sub>2</sub> agonists were also able to induce leukocyte rolling, adhesion, and full recruitment *in vivo* in rat mesenteric venules, by a mechanism involving the release of platelet-activating factor [Vergnolle, 1999]. Whether or not these events are the result of PAR<sub>2</sub> activation on the endothelium or leukocytes still has to be determined. However, several studies have shown that PAR<sub>2</sub> agonists signal to leukocytes (particularly neutrophils and eosinophils). PAR<sub>2</sub> activation resulted in neutrophil activation, cytokine production, and adhesion molecule expression [Howells et al., 1997; Vergnolle et al., 2001b]. PAR<sub>2</sub> agonists induced degranulation and superoxide production in human eosinophils [Miike et al., 2001; Miike and Kita, 2003]. Further studies have shown that PAR<sub>2</sub> agonist can promote the release of eosinophil survival promoting factors (GM-CSF) [Vliagoftis et al., 2001],

implicating further PAR<sub>2</sub> activation as a potential mediator of inflammation/allergic responses in which eosinophils are playing an important role. It is interesting to note that although the edema observed after PAR<sub>1</sub> and PAR<sub>2</sub> agonist intraplantar injection was dependent on a neurogenic mechanism, inflammatory cell infiltration observed in the same tissues was completely independent of sensory nerve activation. This suggests that PAR<sub>1</sub> and PAR<sub>2</sub> agonists are able to induce several of the main features of inflammation (edema, leukocyte infiltration), by different mechanisms. PAR<sub>1</sub> and PAR<sub>2</sub> agonists potentially regulate inflammatory processes by interacting at several levels and with different actors of the inflammatory reaction.

### SENSORY RESPONSE

It has been shown that PAR<sub>1</sub> and PAR<sub>2</sub> agonists can signal directly to primary afferents [Steinhoff et al., 2000]. *In vivo* studies suggest that the signal proteinases are sending to sensory neurons through PARs is not only a neurogenic inflammatory response, but also a nociceptive message. Peripheral administration (into the paw, the colon lumen, or pancreatic ducts) of PAR<sub>2</sub> agonists caused nociceptor activation at the spinal level [Coelho et al., 2002; Hoogerwerf et al., 2001; Vergnolle et al., 2001a; Coelho et al. 2002]. Both intraplantar and intracolonic administration of PAR<sub>2</sub> agonists caused severe and prolonged hyperalgesia [Coelho et al., 2002; Vergnolle et al., 2001a], and the use of PAR<sub>2</sub>-deficient mice has shown that PAR<sub>2</sub> plays an important role in the generation of inflammatory hyperalgesia [Vergnolle et al., 2001a]. In contrast, PAR<sub>1</sub> and PAR<sub>4</sub> agonists exert analgesic properties, increasing nociceptive threshold, and inhibiting inflammatory hyperalgesia and allodynia [Asfaha et al., 2002; Vergnolle et al., 2003]. Taken together, these studies indicate that PARs are implicated in activating or modulating nociceptive pathways and sensory responses to inflammation. (See Vergnolle, this Special Issue of Drug Development Research 59(4):382.)

### REPAIR PROCESS

Many of the cellular effects of thrombin suggest a key role for thrombin in influencing tissue repair and fibrosis [Chambers and Laurent, 2002]. For example, thrombin is a potent mitogen for connective-tissue-producing cells from tissues that are known to promptly develop fibrosis, such as lung, liver, kidney, and skin [Chambers and Laurent, 2002]. PAR<sub>1</sub> agonists have been shown to reproduce thrombin's mitogenic effects on fibroblasts [Trejo et al., 1996]. As well as being a potent fibroblast mitogen, PAR<sub>1</sub> activation also stimulated procollagen production in fibroblasts and smooth muscle cells, and induced a rapid and dramatic

expression of connective tissue growth factor, a novel extracellular matrix signaling molecule [Chambers et al., 1998, 2000]. PAR<sub>1</sub> activation is also mitogenic for endothelial cells at low physiological concentrations [Borrelli et al., 2001]. In vivo studies performed with PAR<sub>1</sub> agonist showed that they enhanced wound healing and neovascularization in experimental animals [Carney et al., 1992]. However, in PAR<sub>1</sub>-deficient animals, no critical role for PAR<sub>1</sub> was found in the setting of skin wound healing [Connolly et al., 1997]. Another of the main features of tissue repair is the neuronal re-growth of damaged tissues. PAR<sub>1</sub> agonists have been shown to inhibit neurite outgrowth from isolated dorsal root ganglia neurons [Gill et al., 1998]. Although this suggests that PAR<sub>1</sub> might play an inhibitory role in nerve regeneration processes, further studies have to investigate this role in vivo. Mitogenic effects have been shown for PAR<sub>2</sub> agonists in endothelial cells [Gill et al., 1998; Yu et al., 1997]. Recombinant tryptase and the PAR<sub>2</sub>-activating peptide SLIGKV exert fibroproliferative effects in human fibroblasts, thus suggesting a role for PAR<sub>2</sub> in repair processes, but also in fibrotic diseases [Frungeri et al., 2002].

#### INVOLVEMENT OF PARs IN INFLAMMATORY PATHOLOGIES

Activation of PARs by selective agonists has been shown to interfere with the regulation of inflammatory response in different tissues or organs. However, the study of the involvement of PARs in pathologies is still hampered by the lack of readily available antagonists for these receptors. The growing availability of PAR-deficient mice is now providing answers concerning the pathophysiology of PARs.

In the lung, PAR<sub>1</sub> agonists induced bronchoconstriction [Cicala et al., 1999a], and PAR<sub>1</sub> expression is increased in alveolar macrophages from smokers compared to healthy subjects, suggesting that PAR<sub>1</sub> plays an important role in the physiopathology of chronic inflammatory airway diseases [Roche et al., 2003]. Activation of PAR<sub>2</sub> using the peptidic agonist SLIGRL-NH<sub>2</sub>, causes relaxation of isolated airway preparations [Chow et al., 2000; Cocks et al., 1999], and protects against bronchoconstrictor challenges in vivo in guinea pigs and rats [Chow et al., 2000; Cocks et al., 1999]. PAR<sub>2</sub> agonist inhalation is able to reduce inflammatory cell infiltration in the lung of rats after exposure to LPS [Moffatt et al., 2002], suggesting a protective role for PAR<sub>2</sub> in lung inflammation. However, the involvement of PAR<sub>2</sub> as a pro-inflammatory agent in the development of allergic inflammation in the airway has recently been unequivocally established [Schmidlin et al., 2002]. In that study, mice deficient

for PAR<sub>2</sub> showed extensive inflammatory reaction compared to wild-type in response to allergic challenge. This suggests, on the contrary, that PAR<sub>2</sub> activation plays a prominent pro-inflammatory role in inflammatory airway diseases (see also Chambers, this Special Issue of Drug Development Research 60(1):29).

In the liver, PAR<sub>1</sub> agonists increased endotoxin-induced liver injury, suggesting a role for PAR<sub>1</sub> activation in promoting inflammatory liver diseases [Copple et al., 2003]. A role for PAR<sub>1</sub> in glomerulonephritis has been suggested by the increased mRNA levels for PAR<sub>1</sub> in patients with crescentic glomerulonephritis. Moreover, PAR<sub>1</sub>-deficient mice showed a reduced level of inflammation and significant protection against crescentic glomerulonephritis, suggesting that PAR<sub>1</sub> plays a pivotal role in kidney inflammatory diseases [Cunningham et al. 2000].

In the mouse bladder, the expression of the four PARs has recently been examined. Upon the induction of acute inflammation, the expression of PAR<sub>1</sub> and PAR<sub>2</sub> was downregulated, while the expression of PAR<sub>3</sub> and PAR<sub>4</sub> was upregulated. These results suggest a differential role for the 4 PARs in bladder inflammation. Whether this role would be pro- or anti-inflammatory still has to be investigated [D'Andrea et al., 2003].

In the gut, where all PARs are widely expressed [Vergnolle, 2000; Vergnolle et al., 2001b], it has been shown that PAR<sub>1</sub>, PAR<sub>2</sub> and PAR<sub>4</sub> local activation induced an acute inflammatory reaction [Cenac et al., 2002; and Vergnolle et al., personal communication], suggesting a role for those 3 receptors in intestinal inflammation. Further studies should be performed to fully investigate the role of those receptors in inflammatory bowel diseases such as ulcerative colitis or Crohn's disease and in infectious gut inflammation. A study by Fiorucci et al. [2001] has shown that in an experimental model of inflammatory bowel disease, daily treatments with PAR<sub>2</sub> agonists were actually protective against the development of chronic inflammation. Although this suggests a protective role for PAR<sub>2</sub> agonists in this particular model, that study [Fiorucci et al., 2002] was investigating systemic effects of PAR<sub>2</sub> activation rather than the effects of PAR<sub>2</sub> activation in colonic tissues. Whether PAR<sub>2</sub> activation in colonic tissues is a pro- or anti-inflammatory event in pathological situations is still an open question (see also Fiorucci and Distrutti, this Special Issue of Drug Development Research 60(1):65).

In an animal model of arthritis, Ferrell et al. [2003] have recently shown that PAR<sub>2</sub>-deficient mice are largely protected against inflammation, suggesting a prominent pro-inflammatory role for PAR<sub>2</sub> in the establishment of arthritic disease. One of the most



challenging questions in this field is still to determine with certitude which endogenous proteinases are responsible for PAR activation in different tissues. Although numerous *in vitro* approaches have revealed some of the proteinases that could potentially activate each of those receptors, scientists still speculate on the pathophysiological circumstances that would lead to sufficient release of proteinases to activate PARs.

### ACTIVATION OF PARs PROTECTIVE AGAINST INFLAMMATION

In most of the tissues where PAR agonists have been administered, an inflammatory reaction takes place, and a protective effect for PAR agonists has also been demonstrated in some circumstances. In the gastric mucosa, activation of PAR<sub>2</sub> by PAR<sub>2</sub>-activating peptides has been shown to be protective against non-steroidal anti-inflammatory drug (indomethacin), or ethanol-induced damage [Kawabata et al., 2001] (see also Nishikawa and Kawabata, this Special Issue of *Drug Development Research* 60(1):9). In the airways, PAR<sub>2</sub> agonists caused relaxation, and protects against bronchoconstrictor challenges [Cocks et al., 1999; Chow et al., 2000]. Moffatt et al. [2002] have shown that intranasal administration of PAR<sub>2</sub> peptidic agonist is protective against LPS-induced airway inflammation, reducing the number of infiltrated leukocytes in bronchoalveolar lavage fluids. In a model of colitis, daily systemic administration of PAR<sub>2</sub> agonist exerts beneficial effects, and increases the survival rate and decreases all the signs of colitis [Fiorucci et al., 2001]. From all these studies, it appears that at least on mucosal surfaces (stomach, colon, and airways), PAR<sub>2</sub> activation could be protective. This protective effect might be explained by the fact that PAR<sub>2</sub> activation in these tissues induces the release of prostaglandins, which are known to be protective for mucosal surfaces. However, other studies report conflicting results at least in the airways, where PAR<sub>2</sub>-deficient mice exhibited a significantly more severe inflammatory response to antigen challenge than wild-type mice. In contrast to the study by Moffatt et al. [2002], this suggests that PAR<sub>2</sub> activation is a pro-inflammatory component of the allergic inflammatory response in the airways.

### CONCLUSIONS

Taken together, all these studies suggest that the role of PARs in inflammatory processes should be considered very carefully, depending on the type of inflammation (bacterial versus allergic, chronic vs. acute) and the tissues (mucosal surfaces vs. other tissues) that are involved. However, it appears clearly that proteinases, through the activation of PARs,

constitute important mediators of all levels of inflammatory processes. Considering the multiple activities of proteinases, and the incertitude on which endogenous proteinases are responsible for PAR activation, the development of PAR-related drugs rather than proteinases inhibitors could be of particular importance in the treatment of inflammatory disorders.

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