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Protease-activated receptors in inflammation, neuronal signaling and pain

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The ability of proteases to regulate cell function via protease-activated receptors (PARs) has led to new insights about the potential physiological functions of these enzymes. Several studies suggest that PARs play roles in both inflammation and tissue repair, depending on the cellular environment in which they act. The recent detection of PARs on peripheral and central neurons suggests that neuronal PARs might be involved not only in neurogenic inflammation and neurodegenerative processes, but also in nociception. Thus, the list of potential roles for PARs has lengthened considerably and their physiological course of action might be much broader than initially anticipated.

There is now substantial evidence that certain proteases, such as thrombin and trypsin, can regulate target cells by cleaving and activating a growing family of G-protein-coupled protease-activated receptors (PARs)[1–8]. The hallmark of this receptor family, now known to possess at least four members (PAR1–4), is the novel mechanism of receptor triggering that involves the proteolytic unmasking of a cryptic N-terminal sequence that, remaining anchored, acts as a 'tethered' receptor-activating ligand (Table 1). For three of the PARs (i.e. PAR1, PAR2 and PAR4, but not PAR3), short synthetic peptides, based on the tethered ligand sequences, have been shown to activate the receptors and therefore mimic the effects of the activating proteases (Table 1). Following the development of receptor-selective PAR-activating peptides (PAR-APs)[9–11], it has been

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Table 1. Protease-activated receptors (PARs): localization, tethered ligands, agonists and possible functions[a]

	PAR1		PAR2		PAR3		PAR4	
	Human	Rat	Human	Rat	Human	Rat	Human	Rat
Localization	Platelets, endothelium, lung	Neurons, GI tract, brain, endothelium	GI tract, leukocytes, endothelium, liver, kidney, lung	Neurons, GI tract, leukocytes, kidney	Lung, endothelium, GI tract, liver, leukocytes	ND[b]	Platelets, lung, GI tract, leukocytes	ND[b]
Tethered ligand	SFLLRN	SFFLRN	SLIGKV	SLIGRL	TFRGAP	ND[b]	GYPGQV	ND[b]
Major activating proteinase	Thrombin	Thrombin	Trypsin, tryptase	Trypsin, tryptase	Thrombin	Thrombin	Thrombin, trypsin, cathepsin G	Thrombin, trypsin
Selective peptide agonists	TFLLR	TFLLR	SLIGKV, SLIGRL	SLIGRL, SLIGKV	– –	– –	GYPGQV, AYPGKF	GYPGKF, AYPGKF
Known physiological role	Platelet activation	–	–	–	ND[b]	ND[b]	Platelet activation	ND[b]
Proposed physiological role	Pro-inflammatory, mucosal protection	Pro-inflammatory, mucosal protection	Pro-inflammatory, mediator of nociception, mucosal protection	Pro-inflammatory, mediator of nociception, mucosal protection	Platelet activation (acts as a cofactor for PAR4) protection	Platelet activation (acts as a cofactor for PAR4) protection	–	Platelet activation

[a]Abbreviations: GI, gastrointestinal; ND, not yet determined.
[b]It can be presumed that the PAR3–PAR4 receptor system will play a role in rat platelets that is comparable to the stimulatory role that has been observed for PAR3–PAR4 in murine platelets.

possible to discern accurately the physiological consequences of activating PARs both *in vitro* and *in vivo*. The discovery of PAR1 resulted from a successful search for the receptor responsible for the cellular actions of thrombin (e.g. platelet aggregation and endothelial cell regulation) and it is therefore widely accepted that PAR1 plays a key physiological role in hemostasis. However, other physiological roles for PAR1 (and by extension, PAR3 and PAR4) and the physiological functions of PAR2 remain to be clarified. An emerging theme has developed implicating a role for these receptor systems in inflammation, and it has been suggested that the PARs might constitute part of the body's defense repertoire in response to injury or invading pathogens[6,8]. Furthermore, new data suggest a key role for PARs on sensory nerves in the regulation of inflammation.

PARs and inflammation

All of the classical 'hallmarks' of inflammation (i.e. pain, swelling, redness, heat and impaired function) have been observed following activation of PARs *in vivo*. Whether the role for PAR activation relates to pro-inflammatory effects, a protective role or both is not yet defined. However, numerous studies suggest that PARs might exert a dual role in inflammation.

Protective role

PAR1 and PAR2 are expressed on endothelial and epithelial surfaces and can regulate the activity of these target cells[3,5,7,8]. *In vivo* studies have demonstrated the ability of PAR2 activation to regulate blood pressure and vascular tone[3]. Moreover, PAR2 has been shown to be upregulated in rat carotid artery in response to balloon-catheter-induced injury[12], which suggests a possible compensatory vasodilator role for PAR2 in response to cardiovascular injury. Further studies by Cirino and colleagues strongly support this hypothesis[13]. These authors showed that after myocardial ischemia–reperfusion injury, infusion of a PAR2-activating peptide significantly improved cardiac function and reduced tissue damage[13]. Another recent study suggests a protective role for PARs in airway epithelia. Both PAR1-APs and PAR2-APs can elicit the relaxation of murine airway preparations through the release of prostaglandin E_2 (PGE_2), thus causing a powerful bronchodilatation[14]. The presence of PARs on epithelial and endothelial cells in a wide variety of tissues, ranging from the vasculature to the kidney and the gastrointestinal tract, might point to a comparable 'anti-inflammatory (protective)' role for epithelial and endothelial PARs in several organs. Furthermore, thrombin, acting via a receptor other than PAR1, was also found to have anti-inflammatory effects in the rat paw, causing a reduction of the edema response induced by intraplantar injection of a PAR1-AP (Ref. 15). The nature of this anti-inflammatory effect of thrombin and the receptor responsible for this effect remains to be determined.

Pro-inflammatory role

Although PAR activation appears to play a protective role via the epithelium and vascular endothelium, many studies have revealed pro-inflammatory effects

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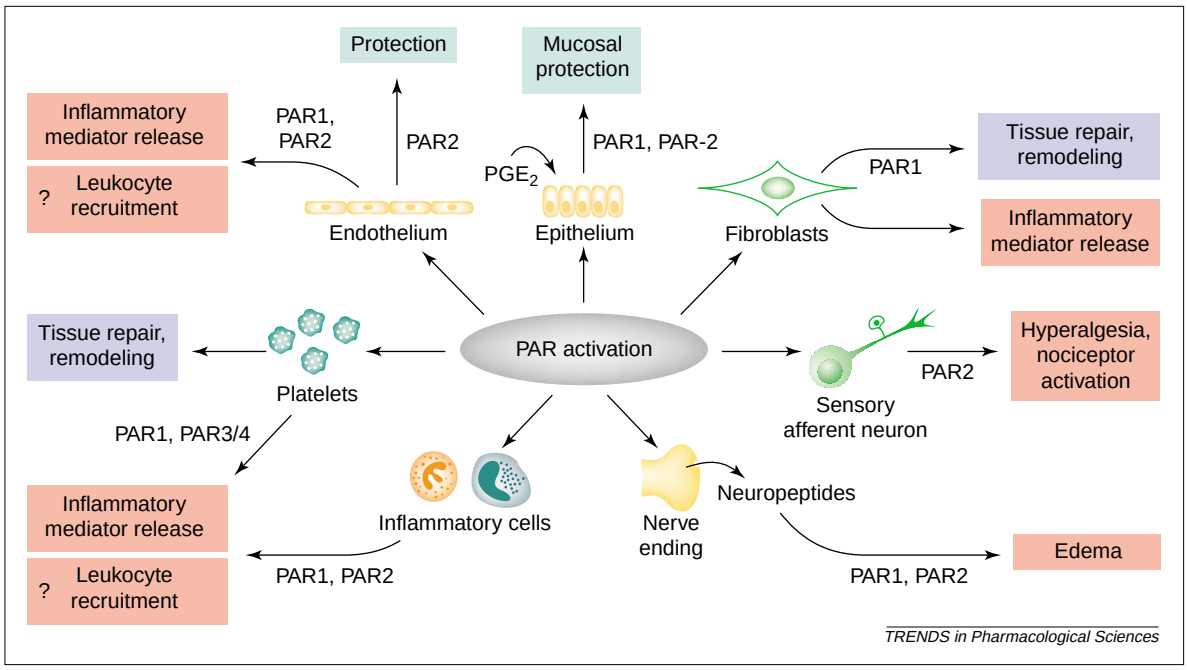

Fig. 1. Inflammatory and protective (repair) effects of protease-activated receptor (PAR) activation. Depending on the cell type, PAR activation can induce protective effects (green) and tissue repair (blue). Alternatively, PAR activation can cause the hallmarks of an inflammatory response, including edema, leukocyte recruitment, hyperalgesia and activation of nociceptive neurons (pink). PARs are widely expressed on endothelium, platelets, inflammatory cells, epithelium, fibroblasts and sensory afferent neurons. Activation of PAR1 and PAR2 on epithelial cells can lead to protection from injury, via a prostaglandin E_2 (PGE_2)-dependent mechanism. Activation of PARs on endothelial cells might also cause a complex response, leading to both protection and inflammatory processes, depending on the vascular bed. On fibroblasts, the activation of PAR1 promotes tissue repair and remodeling by stimulating collagen production and the secretion of growth factors. Activation of fibroblast PARs can also result in the release of prostanoids. On platelets, thrombin-mediated activation of PARs (PAR1, PAR4, PAR3) can lead not only to the release of inflammatory mediators, but also to the secretion of factors (e.g. platelet-derived growth factor) that promote tissue remodeling. On inflammatory cells, activation of PAR1 and PAR2 can also cause the release of inflammatory mediators as well as promoting their recruitment to the site of inflammation. Whether PAR-induced leukocyte recruitment is due to activation of endothelial or inflammatory cells has not yet been defined. PAR1 and PAR2 agonists also directly signal to neurons, inducing the release of inflammatory neuropeptides (e.g. substance P and calcitonin gene-related peptide) that mediate an inflammatory response. Additionally, PAR2 agonists activate nociceptive neurons and induce hyperalgesia.

of PAR agonists. Thrombin is considered the most potent activator of platelets, which play a crucial role in hematologic homeostasis but can also participate in the amplification of inflammatory responses and the recruitment of inflammatory cells[16]. It is now established that PAR1 and PAR4 (in concert with PAR3) are the receptors that mediate thrombin-induced activation of human platelets, whereas PAR3 and PAR4 mediate the response of murine platelets to thrombin[17]. Thus, PARs might play a role in pro-inflammatory effects induced following platelet activation. In addition to its role in the coagulation cascade, thrombin is known to exert a variety of pro-inflammatory effects, most of which appear to be mediated by the activation of PAR1. These include vasodilatation, increased vascular permeability to plasma proteins, and chemotaxis[5,8]. Thrombin, through the activation of PAR1, stimulates various inflammatory cells (e.g. mast cells, lymphocytes and neutrophils) to release mediators such as histamine, eicosanoids and cytokines[5,8], and can induce leukocyte rolling and adherence on post-capillary mesenteric venules[18]. Whether this effect is mediated by PAR1, the PAR3–PAR4 receptor system or perhaps both thrombin targets, located on leukocytes and/or endothelial cells, has yet to be fully investigated.

Several studies also suggest a pro-inflammatory role for PAR2 activation. Activation of PAR2 leads to increased vascular permeability[19,20], smooth muscle relaxation[21,22], systemic hypotension[23,24], bronchoconstriction[25], inhibition of colon rhythmic contraction[26], and leukocyte margination[27] and infiltration[20,27]. PAR2 is highly expressed on inflammatory cells such as neutrophils and mast cells, where it might play a role in cell activation and degranulation[8,28]. Topical exposure of postcapillary mesenteric venules to PAR2-APs induces leukocyte rolling and adhesion[27]. Intraperitoneal administration of the same PAR2 agonists induces extravasation of leukocytes into the peritoneal cavity[27]. These results strongly support the hypothesis that PAR activation can be pro-inflammatory, leading to inflammatory cell recruitment and suggests a dual role for the endothelium not only as a protective element but also as a potential pro-inflammatory factor (Fig. 1).

Role in tissue repair

Another event that characterizes inflammation is the tissue repair process. Thrombin is known to play a role in tissue repair by enhancing neovascularization,

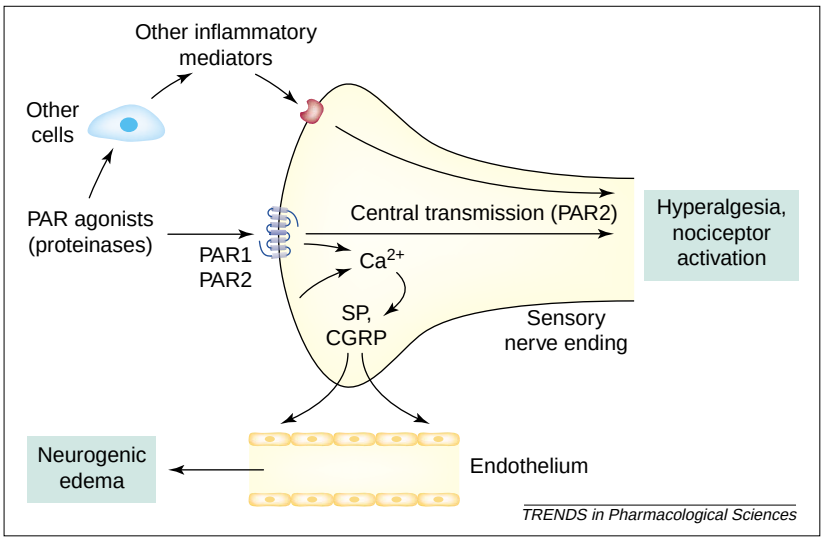

Fig. 2. Protease-activated receptors (PARs) on sensory nerve endings. PAR1 and PAR2 are present on sensory nerves, where their activation causes an intracellular Ca^{2+} signal. This signal results in the release of neuropeptides [e.g. calcitonin gene-related peptide (CGRP), substance P (SP) and possibly neurokinin A] from nerve endings, which in turn interact with their receptors (tachykinin NK_1 and CGRP receptors) on the endothelium to increase vascular permeability and cause vasodilatation (resulting in edema formation). The activation of PAR2 on sensory nerves might also be transmitted centrally, causing the activation of nociceptive neurons at the spinal level and mediating the hyperalgesia observed following PAR2 agonist administration. The possibility that PAR activation on cells other than neurons (e.g. inflammatory cells and endothelium, among others) might induce the release of inflammatory mediators (e.g. prostaglandins, kinins and 5-HT, among others) that could also be responsible for the activation of nociceptive neurons and the hyperalgesic effect cannot be ruled out.

collagen deposition and wound healing[29–31]. Several studies suggest that the effects of thrombin on healing are mediated by PAR1 activation. For example, the stimulatory effects of thrombin on collagen and connective tissue growth factor production by fibroblasts can be reproduced by PAR1-APs (Refs 32,33). Another recent study suggested that thrombin, through the activation of PAR1, participates in tissue remodeling by stimulating the secretion of growth factors from alveolar and bronchial epithelial cells[34]. No effects of PAR2 activation on tissue repair have been reported to date.

PARs as mediators of neurogenic inflammation

Immunohistochemical staining of intestinal tissue has demonstrated that PAR2 is localized not only on endothelial and epithelial cells, but also on enteric neurons[35]. A subsequent study of isolated myenteric neurons showed the simultaneous presence of functional PAR1 and PAR2; activation of either of these receptors resulted in neuronal Ca^{2+} signals[36]. Additional work revealed that PAR1 and PAR2 are also expressed on primary spinal afferent neurons, where their activation causes rapid intracellular neuronal Ca^{2+} mobilization[37]. In dorsal root ganglia, more than a third of neurons expressing PAR2 also coexpress the inflammatory neuropeptides, calcitonin gene-related protein (CGRP) and substance P (SP). By measuring CGRP and SP release from superfused spinal dorsal horn, urinary bladder and atrium, it has been shown that PAR2 agonists (e.g. PAR2-APs and trypsin) can stimulate the release of CGRP and SP from the central and peripheral projections of spinal afferent neurons in a Ca^{2+}-dependent manner[37] (Fig. 2). Because CGRP and SP are known to cause edema by stimulating arteriolar vasodilatation and inducing endothelial gap formation to allow plasma extravasation, it became important to establish whether SP and CGRP, released by peripheral nerve endings in response to PAR activation, might be responsible for the PAR-induced inflammatory response. Therefore, the PAR-induced edema response was re-examined in a rat paw model of peripheral inflammation, with the working hypothesis that neuronal PARs might play a role in the inflammatory response observed after PAR agonist treatment. When injected into rat paws, PAR1 and PAR2 agonists caused a severe edema that lasted for several hours and was accompanied by an intense granulocyte infiltration[15,20,37]. This PAR-mediated inflammatory response was not dependent on mast cell activation, prostaglandin production or nitric oxide release[15,20]. The hypothesis that neuronal PARs play a role in this response was supported by the observation that both PAR1-AP- and PAR2-AP-induced inflammatory edema could be reduced significantly by treatment with a tachykinin NK_1 receptor antagonist or by ablating sensory afferent C-fibers with capsaicin (N. Vergnolle *et al.*, unpublished and Ref. 37). The PAR2-AP-induced inflammatory edema was also significantly reduced in rats treated with a CGRP receptor antagonist[37]. Another study has shown that the PAR2 agonist-induced bronchoconstriction in guinea-pig airways was mediated, at least in part, by a neural mechanism[25]. Taken together, these recent studies have revealed that the local release of proteases (e.g. mast cell tryptase in humans) can, in principle, cause inflammation by a neurogenic mechanism. Because PARs are expressed on sensory afferent neurons, proteases might act directly on sensory nerve endings by a Ca^{2+}-dependent mechanism to induce the release of neuropeptides (SP and CGRP) that are responsible for the PAR-induced edema. However, it is possible that proteases activate PARs present on other cells (e.g. inflammatory cells and endothelium, among others) to induce the release of inflammatory mediators that contribute to neurogenic inflammation (Fig. 2). Although ablation of sensory nerves and the administration of neuropeptide receptor antagonists markedly diminished PAR-AP-induced edema, these treatments did not completely abolish edema, particularly during the early time-points. Furthermore, these treatments did not reduce PAR-AP-induced granulocyte infiltration[37]. These results suggest that there is also a non-neurogenic component of PAR-AP-induced inflammation and thus, endothelial cells, inflammatory cells, platelets or vascular smooth muscle cells, which also express PAR1 and PAR2, might also participate in PAR activation-induced inflammation (Fig. 1).

PARs and neurons in the gut

The activation of neuronal PARs in the intestine might be of particular importance for several reasons. Intestinal tissues are particularly exposed to the proteases known to activate PARs (e.g. thrombin, trypsin and tryptase) in both physiological and pathophysiological conditions. The enteric nervous system plays an essential role in the reflex regulation of intestinal motility, secretion, transport and protection. As mentioned above, PAR1 and PAR2 are

expressed on guinea-pig myenteric neurons that also release neurotransmitters (e.g. SP, vasoactive intestinal peptide and nitric oxide)[36]. Whether activation of these enteric neuronal PARs causes the release of certain neurotransmitters is not yet known. Considering the effects of PAR2 agonists on spinal afferent neurons (release of SP and CGRP), it can be hypothesized that the activation of PARs on myenteric and submucosal neurons might cause the release of neurotransmitters known to participate in the regulation of intestinal motility, secretion and transport or even to facilitate mucosal protection. In keeping with this hypothesis, application of PAR2 agonists (trypsin and PAR2-APs) to the contraluminal side of porcine ileum has recently been shown to induce changes in ion transport by a mechanism that is dependent not only on prostaglandin production (the effect is blocked by indomethacin) but also on neuronal activation (the effect is blocked by saxitoxin, a neuronal conduction blocker)[39]. Whether PAR2 agonists act directly on submucosal myenteric neurons to induce changes in ion transport has yet to be determined. However, evidence has been presented that PAR2 is expressed on both cholinergic and noncholinergic submucosal neurons in the porcine ileum.

PARs, pain and nociception

The discovery of functional PARs on sensory afferent neurons raised the possibility that activation of PAR1 or PAR2 on these neurons would lead to central transmission of a signal. The contribution of PAR2 to the central transmission of nociceptive messages and to the induction of hyperalgesia has recently been studied[40–42]. Intraplantar injection of sub-inflammatory doses of PAR2 agonists in rats and mice induces a long-lasting thermal hyperalgesia[41,42]. Compared with the effects of an intraplantar injection of 0.3 μg of PGE_2, which also induces thermal hyperalgesia, the effects of PAR2 agonists were greater and of longer duration. A dose–response study showed that sub-inflammatory doses of PAR2 agonists were able to induce hyperalgesia[41,42]. The induction of a nociceptive message by PAR2 agonists has been demonstrated by measurement of an increase in the expression of the FOS protein, a marker of activity of nociceptive neurons, in the superficial laminae (laminae I and II) of the spinal cord (L4–L5 level) following the intraplantar injection of PAR2-APs (Ref. 41). Another recent study has shown that intracolonic administration of PAR2-AP produces a delayed (10–24 h) rectal hyperalgesia, suggesting a role for PAR2 not only in somatic pain but also in visceral hyperalgesic states[40]. Whether PAR2 agonists act directly on sensory afferent neurons to induce an action potential and thereby stimulate the release of neurotransmitters within the spinal cord remains to be determined. It is also possible that PAR2 agonists act on cells other than neurons, leading to the release of inflammatory mediators (e.g. prostaglandins, kinins and 5-HT, among others) that could be responsible for activation of nociceptors. However, the study by Steinhoff *et al.* has already shown that PAR2 agonists can signal directly to neurons, inducing the release of neuropeptides from central projections of spinal afferent neurons[37]. Taken together, these studies have identified a previously unknown nociceptive pathway activated by PAR2 and have demonstrated the ability of PAR2 agonists to induce hyperalgesia. Moreover, these studies suggest a role for proteases in pain transmission, and suggest that neuronal PARs are potential therapeutic targets in inflammation and pain.

PARs in the CNS

In the CNS, prothrombin and PAR1 are widely expressed on neurons and glial cells, and neuronally derived thrombin can activate PAR1. Although less work has been done to localize PAR2 in the CNS, Smith-Swintosky *et al.* have shown that PAR2 is expressed in the rat hippocampus and that PAR2 agonists can be toxic to cultured hippocampal neurons, which is suggestive of a role for PAR2 in neurodegeneration[43]. PAR1 activation in the CNS appears to have dual effects. On the one hand, PAR1 agonists induce neurite retraction[44] and are neurotoxic. A recent study has also shown that motor neuronal degeneration and death follows PAR1 expression both temporally and topographically in wobbler mice[45]. On the other hand, PAR1 agonists protect neurons from death induced by hypoglycemia and oxidative stress[46,47]. Thus, depending on the circumstances, PAR1 activation in the CNS might contribute both to 'appropriate' as well as 'inappropriate' neuronal cell death. PAR1 agonists have also been shown to stimulate proliferation and shape changes in astrocytes[48], to cause the release of endothelin-1 and nerve growth factor and to inhibit the expression of glutamate receptors. Direct infusion of thrombin into the brain has been shown to reproduce inflammatory signs observed after injury in the CNS. Moreover, an increased amount of thrombin in the brain has also been suggested to be associated with Alzheimer's disease[49]. However, the true physiological functions of PARs and proteases in the CNS remain largely unknown.

Where do the proteases come from?

Although little is known about extrapancreatic trypsin, trypsinogen is released by several cell types (e.g. endothelial cells and epithelial cells)[14,50]. Extrapancreatic trypsin-2 *in vitro* is able to cleave PAR2 (Ref. 51) and thus might cleave PAR2 in cells that are exposed to it. Nonetheless, one of the major obstacles in defining the physiological role of PAR2 is that even if trypsin is able to activate PAR2, as it appears to do in the intestine, trypsin itself is not present in most tissues. Thus, the endogenous enzymes that activate PAR2 remain to be identified.

Tryptase, which constitutes the major protein released during human mast cell degranulation, is able to cleave PAR2 *in vitro*[52] in cells that naturally express the receptor or in cells transfected with the receptor. The observation that tryptase *in vitro* can cleave and activate PAR2 suggests a role for this receptor in inflammatory states in humans that are associated with mast cell degranulation. Because mast cells are closely associated with nerves[53,54], mast cell proteases, including tryptase, are potential candidates for the activation of PAR2 on neurons. However, despite the ability of tryptase to activate PAR2 *in vitro*, the direct activation of PAR2 by tryptase has yet to be demonstrated *in vivo*. Thrombin, which is released during endothelial damage, could have access to neurons and appears to be the most likely agonist to activate neuronal PAR1 *in vivo*.

Concluding remarks

PAR activation can lead to either pro-inflammatory or protective effects. This dual role for PAR activation might depend on the tissues that are exposed to proteases, the nature of the inflammatory stimulus and the time-frame of protease activation. The protective effects of PAR activation show some similarities with the effects of other well-known pro-inflammatory mediators such as substance P or bradykinin, which can induce airway relaxation or cardioprotection[55,56]. Thus, the roles of the PAR system might not differ from the roles for other pro-inflammatory mediators. The discovery that PARs are expressed on neurons adds a new dimension to the potential protective and damaging effects that PAR activation might have in the PNS and CNS. In addition to a role in inflammation, the presence of PARs on neurons and the ability of PAR2 agonists to cause neuronal sensitization and hyperalgesia points to a novel role for proteases and PARs in pain transmission. Whether the different PAR receptor systems will be found to play complementary or distinct roles in the regulation of inflammation, protection and nociception remains an attractive topic for further investigation. The lack of readily available selective and potent receptor antagonists (particularly for PAR2) hampers definitive studies of the involvement of PARs in inflammatory and pain processes. However, the use of mice deficient in genes encoding PARs should provide new insights into the role of proteases and PARs in inflammation and pain.

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Oestrogen and the cardiovascular system: the good, the bad and the puzzling

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The concept that oestrogen replacement therapy is cardioprotective has been challenged recently by the negative results of randomized clinical trials in coronary heart disease. These data have come at a time of rapid advances in our understanding of the cellular mechanisms of oestrogen. In particular, the cloning of the classical oestrogen receptor (ERα), the identification of a novel ER isoform (ERβ), the availability of specific ERα and ERβ knockout mice models, and the elucidation of receptor functions and signalling pathways linked to non-genomic actions of oestrogen are helping to unravel this complex biology. In this article, these advances will be discussed with particular emphasis on the regulation of nitric oxide synthesis by oestrogen. Furthermore, the puzzling issues that have emerged and the potential for development of novel and specific therapeutic approaches will be highlighted.

Premenopausal women have a low incidence of coronary heart disease, which rises rapidly after the menopause towards incidence levels observed in men. This striking gender difference in the incidence of coronary heart disease has spawned a plethora of studies aimed at unravelling the role of female sex steroids in the determination of cardiovascular risk and pathogenesis. A large number of retrospective and cross-sectional studies show that women who receive oestrogen replacement therapy (ERT) have a substantially lower incidence of coronary heart disease than non-treated women. The consequent notion that oestrogen prevents coronary heart disease is supported by a vast body of evidence from studies in experimental animals and mechanistic studies in humans[1]. Maintenance of a favourable lipid status is believed to be a major protective effect but other mechanisms, including enhancement of endothelial function and inhibition of cell proliferation, are also likely to contribute[2]. However, to date, only one randomized prospective controlled clinical trial designed specifically to examine the effects of postmenopausal ERT on cardiovascular risk has been published in full. Surprisingly, the Heart and Estrogen–progestin Replacement Study (HERS)[3] showed no overall benefit of ERT. In fact, a trend towards a delayed benefit after two years of therapy was offset by a significant 50% increase in cardiovascular events in the first year on

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