Forum Minireview

Physiology and Pathophysiology of Proteinase-Activated Receptors (PARs): PAR-2 as a Potential Therapeutic Target

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Abstract. PAR-2 is the second member of the family of proteinase-activated receptors activated by trypsin, tryptase, and several other serine proteinases. In order to evaluate the therapeutic potential for PAR-2, we have performed studies on PAR-2-mediated signal transduction and investigated the effects of PAR-2 gene deficiency in disease models. In addition to the G-protein-coupled receptor-mediated common signal transduction pathways, inositol 1,4,5-trisphosphate production and mobilization of Ca^{2+} , PAR-2 can also activate multiple kinase pathways, ERK, p38MAPK, JNK, and IKK, in a cell-type specific manner. The studies using PAR-2-gene-deficient mice highlighted critical roles of PAR-2 in progression of skin and joint inflammation. We also describe the development and evaluation of potent and metabolically stable PAR-2 agonists in multiple assay systems both in vitro and in vivo. The structure-activity relationship analysis indicated the improved potencies of furoylated peptides. Furthermore, the resistance of the furoylated peptide against aminopeptidase contributed to the highly potent and sustained effects of the peptide in vivo. These studies suggest the potential therapeutic importance of PAR-2 in inflammatory diseases. Also, the PAR-2-gene-deficient mice and the potent and metabolically stable agonists are shown to be useful tools for evaluating the potency of PAR-2 as a therapeutic target.

Keywords: proteinase-activated receptor (PAR)-2, mitogen-activated peptide kinase, PAR-2-gene-deficient mice, PAR-2-activating peptide

Introduction

Identification of the family of proteinase-activated receptors implies a key role for tissue and systemic serine proteinases, not only as protein-degrading enzymes, but also as potential G-protein-coupled receptor (GPCR) activators that transmit extracellular stimuli into intracellular signalling events (reviewed in refs. 1, 2). The second member of this family of receptors, proteinase-activated receptor-2 (PAR-2), which was originally identified as a receptor activated by trypsin (3), has drawn attention by its prospective functions distinguished from other PARs activated by thrombin. Following the discovery of PAR-2, extensive studies

have revealed that this receptor is expressed in a wide variety of epithelial and endothelial tissues (4) that have important physiological roles in regulating a wide range of biological systems, including the cardiovascular system (5) and gastrointestinal system (6), skin function (7), respiratory system (8), and peripheral nerves (9).

Although there is accumulating evidence for the involvement of PAR-2 in certain disease states, the determination of therapeutic effects and usefulness of PAR-2-targeted drugs in human disease should await the future development of specific antagonists. In this part of the review, we will present our studies, to evaluate the therapeutic potential for PAR-2, in PAR-2-mediated cellular signal transduction and in physiological responses of PAR-2-gene-deficient mice. We also will present recent developments and use of potent PAR-2 agonists as a tool to examine biological functions of

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the receptor.

PAR-2-mediated signal transduction

Activation of PAR-2 elicits diverse cellular responses, including cell proliferation (10, 11), differentiation (7), and production and release of proteins such as, interleukin-6 (IL-6), granulocyte macrophage-colony stimulating factor (GM-CSF) (12), and eotaxin (13). However, signal transduction pathways leading to the diverse cellular responses remain uncharacterized. In a number of different cell types, PAR-2 effectively couples to the Gq/11 family G-proteins to stimulate phospholipase C (PLC) activation, resulting in generation of second messengers, inositol 1,4,5-trisphosphate (IP₃), and diacylglycerol (DG), which further trigger mobilization of Ca²⁺ and activation of protein kinase C (PKC) (14, 15). In addition to this common signal transduction pathway, activation of various kinase cascades, which are suggested to be key regulators of inflammatory responses, has been demonstrated. In our observation, PAR-2-mediated extracellular signal regulated kinase (ERK) activation in NCTC2544 cells was insensitive to a PKC inhibitor (Fig. 1A). On the other hand, PAR-2-mediated activation of other members of the

mitogen-activated protein kinase (MAPK) family, c-Jun N-terminal kinase (JNK) and p38MAPK, were partially regulated by PKC (Fig. 1: B and C). Similarly, activation of nuclear factor kappa B (NFκB) and its upstream regulating kinase, I kappa B kinase (IKK), were also found to be partially PKC-dependent (16). Previous studies reported selective activation of p38MAPK, whereas no activation of JNK was observed in rat aortic smooth muscle cells (17). This may imply PAR-2 transmits important cellular responses through activation of multiple kinase pathways, ERK, JNK, p38MAP kinase, and IKK, in a cell-type specific manner; and the various PKC subtypes may regulate different cellular responses. However, mechanisms for the activation of these kinase pathways need to be examined in more detail at the molecular level.

Physiological studies using PAR-2-gene-deficient mice

The effect of PAR-2 gene deletion was first described in vascular systems. Activation of PAR-2 by trypsin or PAR-2-activating peptides mediates relaxation of isolated rat aorta by an endothelium-dependent, nitric oxide (NO)-mediated mechanism (18). A study utilizing PAR-2-gene-deficient mice confirmed the *in vivo* function for PAR-2 in mediating vasodilation (5). In addition to regulating vascular tone, another important role for PAR-2 in vascular inflammation has been described. In endothelium, increased expression of the leukocyte adhesion molecule, P-selectin, was stimulated by PAR-2 activation, and this in turn positively regulated leukocyte accumulation, causing chronic inflammation (19). This was evidenced in a PAR-2-gene-deficient mouse study using intravital microscopy (20). These studies, combined with the early observation of increased PAR-2 mRNA expression following cytokine pre-treatment of vascular endothelial cells (21), strongly suggested a role for PAR-2 in the integration of vascular inflammation. Similar mechanisms may be responsible in our observation on contact-induced skin inflammation, which was abrogated in PAR-2-gene-deficient mice (22). Another line of evidence for PAR-2 in association with chronic inflammation was demonstrated in an adjuvant monoarthritis model (23). Disruption of the PAR-2 gene results in ablation of chronic arthritis both at levels of joint swelling (Fig. 2A) (23) and in histological evaluation. Up-regulation of PAR-2 in inflamed synovial tissues was also demonstrated in the arthritis, suggesting the pro-inflammatory effects of PAR-2 mainly at the local tissues. However, involvement of PAR-2 in immune responses should also be considered, as the effect of PAR-2 on dendritic cell development (24). Other reports using PAR-2-gene-deficient mice demon-

Fig. 1. Effect of PKC inhibitor Gö6983 on activation of ERK (A), JNK (B), and p38MAPK (C) in NCTC2544 cells expressing PAR-2. Cells were stimulated with either 30 nM trypsin, 100 nM PMA or 25 ng/ml TNFα; and activation of kinases were determined after following time points: at 5 min (for ERK (A)) and at 30 min (for JNK (B) and p38MAPK (C)). Gö6983 (10 μM) was added 15 min prior to the stimulation. Modified from Ref. 16 with permission.

Fig. 2. (A) Effect of PAR-2 gene disruption in adjuvant arthritis. Comparison of the response to induction of adjuvant arthritis in PAR-2^{+/+} mice (closed triangle), PAR-2^{-/-} mice (open circle), and heterozygous mice (closed square). (B) Joint swelling induced by intra-articular injection of SLIGRL (closed square), ASKH95 (closed circle), or control peptide (closed triangle) in PAR-2^{+/+} mice and ASKH95 (open circle) in PAR-2^{-/-} mice. Modified from Ref. 23 with permission.

strated important roles of PAR-2 in hyperalgesia (25) and intestinal inflammation (26), which may coordinate neurogenic inflammation and pain. Further application of PAR-2-gene-deficient mice in other physiological systems will provide direct evidence for important physiological roles of PAR-2 in other tissues and disease states. In addition to evaluation of biological functions of the receptor, the PAR-2-gene-deficient mice, and tissues and cells derived from them, can be utilized to assess the selectivity of agonists and antagonists.

PAR-2 agonists and antagonists

The synthetic agonist peptides mimicking the tethered ligand of PAR-2, SLIGKV-OH (human), SLIGRL-OH (mouse/rat) (3, 27), and their amidated forms SLIGKV-NH₂ and SLIGRL-NH₂ (14, 18) were also shown to be

Table 1. EC₅₀ or ED₅₀ values and the relative potency of PAR-2-activating peptides

	EC ₅₀ or ED ₅₀ (Relative potency)		
	Ca ²⁺ response in HCT-15 cells	Salivation	
		Amastatin (-)	Amastatin (+)
SLIGKV-OH	22.8 (1)	32.9 (1)	31.7 (1)
SLIGRL-OH	7.87 (2.90)	9.95 (3.31)	7.86 (4.03)
SLIGKV-NH ₂	5.05 (4.51)	11.4 (2.88)	3.10 (10.2)
SLIGRL-NH ₂	3.13 (7.28)	2.84 (11.6)	0.250 (127)
2f-LIGKV-OH	1.68 (13.6)	0.840 (39.2)	0.690 (45.9)
2f-LIGRL-OH	1.21 (18.8)	nd	nd
2f-LIGKV-NH ₂	0.318 (71.7)	0.070 (470)	0.0600 (528)
2f-LIGRL-NH ₂	0.253 (90.1)	0.0300 (1100)	0.0400 (793)

EC₅₀ (μM) and ED₅₀ (μmol/kg) were calculated in each assay system. The relative potency shown in parentheses was calculated by an equation, (EC₅₀ or ED₅₀ of SLIGKV-OH)/(EC₅₀ or ED₅₀ of each peptide). nd, not determined. Modified from Ref. 28 with permission.

able to activate the receptor without enzymatic cleavage. In addition to the original PAR-2 activating peptides, a series of potent PAR-2 agonists, by modification of the native PAR-2-activating peptides substituted with 2-furoyl on the N-terminal serine, have been described recently. The initial identification was achieved by Plevin's group who showed that 2-furoyl-LIGKV-OH (ASKH95) exhibited potent PAR-2 agonist activity in the cellular inositol phosphate assay, and strikingly, the intra-articular injection of the modified peptide resulted in sustained joint swelling in mice (Fig. 2B) (23). In the follow-up studies, detailed structure-activity relationships of a series of 2-furoyl agonist peptides have been examined in multiple assays, cellular Ca²⁺ mobilization, rat mesenteric artery relaxation, and saliva secretion in mice, demonstrating that 2-furoyl-LIGRL-NH₂ has the highest PAR-2-activating potency (Table 1) (28). Additionally, the increased resistance of furoylated peptides to a peptide-metabolizing enzyme, aminopeptidase, resulted in dramatic increase in PAR-2-activating potency in vivo (Fig. 3B) (28). Consistent with this observation, an independent study also described a similar modified peptide, 2-furoyl-LIGRLO-NH₂, as a potent PAR-2-activating peptide (29). It is not clear whether the addition of C-terminal ornithine alters the PAR-2 agonistic activity; nevertheless, it is clear that 2-furoyl peptides are the most potent agonistics currently available, which are useful in for evaluating PAR-2 function.

As for the PAR-2 antagonists, currently very little information is available. In the studies on structure-activity relationships (SARs) of PAR-1-activating peptides, substitution of the N-terminal serine with a neutral hydrophobic N-acyl group (e.g., *trans*-cinnamoyl group) provided peptides that were partial agonists or antago-

Fig. 3. Structure of 2-furoyl-LIGRL-NH₂ (A) and metabolic stability of PAR-2-activating peptides in vitro (B). The peptide (50 μ g/ml) was incubated with aminopeptidase (0.2 U/ml) for 0–60 min in the absence [SLIGRL-NH₂ (open circle), 2-furoyl-LIGRL-NH₂ (open triangle)] or presence [SLIGRL-NH₂ (closed circle), 2-furoyl-LIGRL-NH₂ (closed triangle)] of amastatin (2.5 μ M). Data show the mean values of duplicate experiments. Modified from Ref. 28 with permission.

nists for PAR-1 (30). In contrast, the similar modification on PAR-2 agonist peptides provided peptides with agonistic activity, for example, *trans*-cinnamoyl-LIGRLO-NH₂ (31). In a study on peptides derived from PAR-2 agonist peptides, two peptides, FSLLRY-NH₂ and LSIGRL-NH₂, have been described to antagonize trypsin-induced PAR-2 activation (32). However, these peptides are unable to antagonize PAR-2-activating peptides, suggesting these peptides bind to the receptor-trypsin association site, although its exact mechanism is not clear. So far, currently no clear antagonists for PAR-2 have been reported; however, the SARs on PAR-2 agonist peptides and related peptides may provide useful information for future development of PAR-2 antagonists.

PAR-2 as a therapeutic target

As presented, a number of studies both in vitro and in vivo implicate PAR-2 in the mediation of inflammatory responses. The mechanisms of PAR-2-elicited inflammation consist of multiple cascades, including increase of vascular permeability, cytokine production, and

expression of adhesion molecules that further lead to leukocyte accumulation (19). Additionally, PAR-2-stimulated secretion of prostanoids (33) and NO (34) may enhance the inflammatory responses, while activation of PAR-2 in nerve endings induces the release of calcitonin gene-related peptide (CGRP) and substance P (35). Therefore, the future development of PAR-2 antagonists can be therapeutically beneficial for treatment of a number of inflammatory diseases such as allergic dermatitis, rheumatoid arthritis, asthma, inflammatory bowel disease, and neurogenic pain.

Several important physiological roles of PAR-2 in maintaining homeostatic biological functions have also been described in several organs such as secretory glands (36), kidney (37), and respiratory systems (8). Therefore, in these situations, loss of PAR-2 expression and dysfunction of the receptor may cause diseases. Potent and stable PAR-2 agonists such as 2-furoyl-peptides can be powerful tools to explore these possibilities.

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