Inflamm. Res. 56, *Supplement 3* (2007) S499 – S502
1023-3830/07/03S499-4
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Inflammation Research

Mini-Paper 5

Kallikrein-mediated activation of PARs in inflammation and nociception.

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Abstract. Kallikreins (KLKs) are secreted serine proteinases identified in a variety of cancers and in settings of inflammation. We have shown that KLKs *in vitro* can activate proteinase-activated receptors (PARs), a family of G-protein coupled receptors associated with inflammation. KLK14 activated both of PARs 2 and 4 and prevented thrombin from activating PAR_1. On the other hand, KLK6 was a specific activator of PAR_2. In an *in vivo* mouse inflammation model, kallikrein administration caused an oedema response comparable in magnitude and timing to that generated by trypsin. The oedema was accompanied by a decreased threshold of mechanical and thermal nociception. Our data demonstrate that by activating PARs 2 and 4 and by inactivating PAR_1, widely-expressed kallikreins, like KLKs 6 and 14, may play a role in regulating the inflammatory response and pain perception.

Introduction

Proteinase activated receptors (PARs 1 to 4) comprise a unique family of four G-protein coupled cell surface receptors (1-3). Proteolytic cleavage within the extracellular N-terminus by certain proteinases reveals a tethered ligand that binds to the extracellular receptor domains to initiate cell signalling (1-3). Proteinases that activate PARs range from enzymes produced by inflammatory cells to proteinases from epithelial cells and neurons. Other proteinases that cleave PARs downstream of the N-terminal tethered ligand sequence disable the receptors for further proteolytic activation (1-3).

Many of the proteinases that can activate PARs have been identified. However the endogenous enzymes responsible for activating PAR_2 in settings other than the gastrointestinal tract where trypsin can activate PAR_2 are unknown (1-3). Moreover, little is known about the proteinases that can inactivate either this receptor or other members of the PAR family in many settings. It is of considerable interest to identify the proteinases that activate and/or disable PARs, in view of the emerging role that these receptors can play in diseases such as asthma, arthritis, inflammatory bowel disease, cancer and neuronal inflammation (1, 4-6).

Tissue kallikreins comprise a large family of secreted serine proteinases with tryptic or chymotryptic activity (7). These enzymes are widely expressed and up-regulated in many diseases such as ovarian cancer and multiple sclerosis. The mechanisms whereby this enzyme family regulates cellular function are not clear. We hypothesized that kallikreins can cleave PARs initiating an inflammatory response in many (patho)physiological settings.

Methods

To test our hypothesis for kallikreins 5, 6 and 14 we used (8, 9): (1) PAR-dependent calcium signalling responses in cell lines expressing PARs 1, 2 and 4, and in isolated human platelets, (2) a vascular ring vasorelaxation assay using tissues from rats and both wild-type and PAR_2 null mice, (3) a PAR_4-dependent rat and human platelet aggregation assay, and (4) *in vivo* models of inflammation and nociception employing wild-type and PAR_2 / PAR_1 null mice.

Additionally, we developed analytical tools to monitor the presence and enzymatic activity of these enzymes in biological samples where PARs are abundant using: (1) covalent activity-based probe

western blot detection, and (2) activity-based probe ELISAs.

Finally, we studied the specificity of kallikreins to target cell membrane PARs in cultured human breast-cancer-derived MDA-MB-231 cells that express PARs 1, 2 and 4, along with other receptors [10]. The method comprises a proteomic identification of the cell surface peptides released upon incubation with kallikreins.

Results

Calcium signalling, platelet aggregation and vasorelaxation. The ability of KLKs 5, 6 and 14 to cleave and activate PARs 1, 2 and 4, was evaluated in human and rat PAR-expressing cells and in an *in vitro* PAR_2-dependent aorta relaxation bioassay. All kallikreins triggered a PAR_2-mediated cellular calcium response (example in Fig. 1), but with different potencies. However, only kallikrein 14 was able to activate PAR_4 either in PAR_4-expressing cells (calcium) or in human and rat platelets (calcium signalling; aggregation, Fig. 2). In contrast, KLK14 selectively dis-armed human PAR_1, preventing its activation by thrombin (Fig. 1). In the vascular assay, all kallikreins triggered a PAR_2-mediated relaxation that was absent in tissues from PAR_2-null mice.

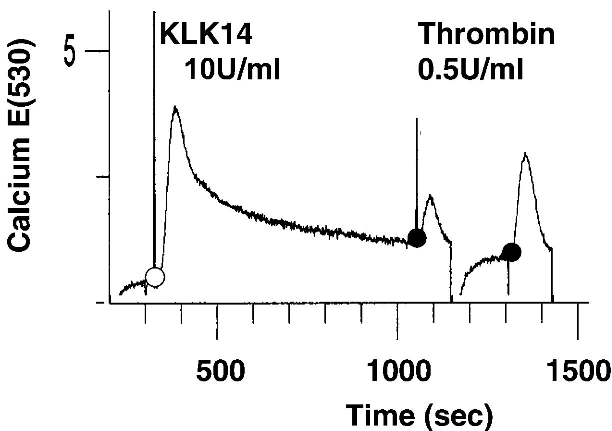

Fig 1. Kallikrein 14 has a complex action in HEK293 cells that express PARs 1 and 2; mainly decreasing the PAR_1 response to proteinases like thrombin, as well as activating PAR_2. Adapted from reference 8.

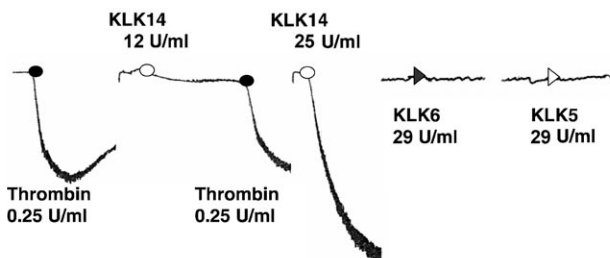

Fig. 2. Kallikrein 14-mediated rat platelet aggregation. Adapted from reference 8.

Inflammation/nociception models. Intraplantar administration of kallikrein 14 in wild-type mice resulted in an oedema response similar to the one caused by trypsin that did not cause oedema in PAR_2 null mice. However, in PAR_2 null animals KLK14 administration resulted in an even greater oedema response, along with a decreased threshold of thermal nociception, which to a lesser extend was also observed in wild-type mice (Fig. 3).

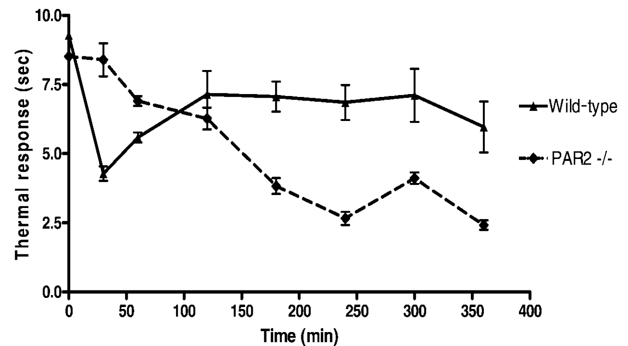

Fig. 3. Kallikrein 14 injection results in increased pain perception upon thermal stimulation.

Cell surface targets of KLK14. Treatment of MDA-MB-231 cells with KLK14 resulted in the fragmentation of more than a hundred target molecules identified by proteomic analysis, including matrix-related molecules (e.g. thrombospondin-1), cell surface components (e.g. receptors) and secreted proteins (e.g. Insulin-like growth factor-binding protein 7 IGFBP-7). Amongst the cell surface molecules, PAR_2 was succesfuly identified thereby confirming biochemically, the ability of KLK14 to target cell surface PARs (Table 1).

Discussion

Our main finding was that signalling via human, rat and murine PARs, and stimulation of inflammation with increased nociception *in vivo*, can be regulated by human kallikreins. Unlike tryptase, but like trypsin family members, the PAR_2-regulating actions of kallikreins, which have a wide tissue distribution, are not restricted by receptor glycosylation [11, 12]. The ability of KLKs to modulate PARs can now be added to their action on other substrates (e.g. kininogens, extracellular matrix, growth factor precursors or

Table 1. Mass spectral protein identification upon treatment of MDA-MB-231 cells with KLK14. Protein hits with probabilities over than 94 % are included in the table.

Proteins common to kallikrein-treated and non-treated samples	Proteins unique to kallikrein-treated samples
49 kDa protein	13 kDa protein
78 kDa glucose-regulated protein precursor	89 kDa protein
Actin, cytoplasmic 1	Coagulation factor V
ALB protein	Complement C4-A precursor
CD44 antigen isoform 4 precursor	Dynein heavy chain, cytosolic
CDNA FLJ32800 fis, clone TESTI2002544	Galectin-3-binding protein precursor
CYR61 protein	Gelsolin precursor
Elongation factor 1-alpha	Hypothetical protein WDR60
Fibrinogen beta chain precursor	Insulin-like growth factor-binding protein 7 precursor (IGFBP-7)
FLJ00173 protein (Fragment)	Interferon-induced protein with tetratricopeptide repeats 1
Glyceraldehyde-3-phosphate dehydrogenase	PREDICTED: similar to 60S ribosomal protein L6 (TAX-responsive enhancer element binding protein 107) (TAXREB107) (Neoplasm-related protein C140) isoform 1
H3 histone family, member M	Probable ATP-dependent RNA helicase DDX41
Hemoglobin alpha subunit	**Proteinase-activated receptor 2 precursor (PAR_2)**
Histone H1.2	Receptor-type tyrosine-protein phosphatase F precursor
Histone H2A type 1-H	Serine/threonine-protein kinase PLK1
Histone H2B.c	Splice Isoform 1 of Periostin precursor
Hypothetical protein DKFZp761K0511	Splice Isoform 2 of ATP-binding cassette sub-family F member 1
hypothetical protein LOC65250	Splice Isoform 2 of Basigin precursor
Kinesin-like protein 2	Splice Isoform 2 of Structural maintenance of chromosomes 4-like 1 protein
POLRMT protein	Splice Isoform EWS of RNA-binding protein EWS
PREDICTED: AHNAK nucleoprotein 2 isoform 1	Thrombospondin-1 precursor *
PREDICTED: hypothetical protein XP_943141	Transcriptional repressor protein YY1
PREDICTED: similar to Nucleophosmin	
RB-binding protein	
RcTPI1 (Fragment)	
Splice Isoform 1 of Heat shock cognate 71 kDa protein	
Splice Isoform 1 of Tubulin alpha-2 chain	
Splice Isoform 2 of Putative quinone oxidoreductase	
Splice Isoform B of Golgi SNAP receptor complex member 2	
Splicing factor, arginine/serine-rich 4	
Testis-specific serine/threonine-protein kinase 6	
Thrombospondin-1 precursor *	

* Thrombospondin-1 spectrum identification analysis revealed that differentially cleaved fragments were identified in non-treated and treated samples, most probably due to degradation from different proteinases.

binding proteins) thought to enable these enzymes to affect tumour survival, metastasis and angiogenesis [7, 9].

Our focus was principally on PAR_2, known to play an important role in inflammation and nociception (5) and our preliminary data clearly implicate KLKs as inflammatory mediators. However, the results indicate that PAR_2 is not the only target for the inflammatory and nociceptive actions of KLK14. Continuing work aims to identify the PAR and non-PAR kallikrein targets involved in the inflammatory/nociceptive responses.

In terms of the actions of KLKs *in vivo,* the enzymatic dis-arming/inhibition of PAR_1 by KLKs may be as important as its activation [9]. For example, by preferentially activating human platelet PAR_4 whilst dis-arming PAR_1, KLK14 would promote the

release of platelet endostatin and suppress the release of platelet VEGF in inflammatory settings.

The ability of released KLKs to regulate inflammation and nociception *in vivo* will depend on the levels of active enzymes produced in a given setting. In this regard, our newly developed activity-based probe tools will enable us to determine the proportion of active enzyme relative to the surprisingly high levels of ELISA-measured kallikreins known to be present in many tissue extracts and biological fluids.

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