Kallikrein-related peptidase signalling via proteinase-activated receptors

Aikaterini Oikonomopoulou
Doctor of Philosophy, 2008
Department of Laboratory Medicine and Pathobiology
University of Toronto

ABSTRACT

The family of human kallikrein-related peptidases (KLKs) numbers 15 serine proteinases implicated in tumour progression. Despite the wide tissue distribution of KLKs and the numerous reports of their differential expression in pathological settings, the signalling mechanism(s) whereby these enzymes regulate tissue function are not yet known. Further, knowledge of the levels of their activity, as well as of their potential endogenous targets, has only been extracted from in vitro studies and cell culture systems.

We hypothesized that KLKs can trigger tumour signalling via proteinase-activated receptors (PARs), a family of G-protein-coupled receptors. To test our hypothesis, we evaluated the ability of KLKs 5, 6, and 14 to activate or prevent signalling via PARs 1, 2, and 4 in cells and tissues expressing these receptors. Further, we used a novel activity-based probe approach, coupled with conventional immunoassay (ELISA), to determine the abundance of active KLK6 relative to total immunoreactive KLK6 in cancer-related biological fluids.

We concluded that KLKs can regulate multiple signalling pathways triggered by PARs 1, 2, and 4, resulting in calcium release, platelet aggregation and vascular relaxation, and they can cause murine inflammation. Further, our activity-based ELISA demonstrated the presence of active KLK6 in ovarian cancer ascites fluids and cancer cell supernatants. We, therefore, suggest that tumours can produce active KLKs, which can potentially control tumour behaviour by regulating PAR activity.
ACKNOWLEDGEMENTS

Completing a PhD thesis is not a matter of receiving another degree to hang on the wall but rather a justification of being able to deal with difficult questions and generate problem-solving ideas. It is a path which requires wise mentorship and guidance, as well as putting into practice your intuition and self-confidence. I, therefore, consider myself extremely lucky that I was able to have the best mentorship from two supervisors, Dr. Morley D. Hollenberg and Dr. Eleftherios P. Diamandis, with different scientific backgrounds, both being experts in their field. It is the successful bonding of these two different fields that ensured my adventurous but fruitful journey during the past 4 years.

I would also like to extend my appreciation to Dr. Harry Elsholtz, my graduate coordinator, and my committee advisors Dr. Bharati Bapat, Dr. Joanne McLaurin, and Dr. Alex Romaschin for their useful advices and scientific discussions throughout this project, as well as to Dr. Christopher Power and Dr. Martin McGavin for valuable input on the content of my thesis. I am also very grateful to Antoninus Soosaipillai, Dr. Mahmoud Saiffedine, Dr. Kristina K. Hansen, Dr. Amos Baruch, Dr. Nathalie Vergnolle, Kevin Chapman, Dr. Rithwik Ramachandran, and Eric Huyan for sharing their knowledge and expertise with me. Also, I would like to thank Linda Grass, Tammy Earle, Bernard Renaux, and Dr. John (Zhenguo) Yu for their help and time. Furthermore, I would like to thank the members of both laboratories and my friends for sharing ideas and moments in and outside the lab.

Above all, I would like to express my sincere gratitude to my father, mother, sister, and my husband, who all led my way from undergraduate to graduate university, managed to put up with my ambitions and constant lack of time, and supported me all the way to the completion of this thesis.
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<tr>
<td>19q13.3-13.4</td>
<td>Chromosome 19, long arm, cytogenetic band 13.3-13.4</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid(s)</td>
</tr>
<tr>
<td></td>
<td>Note: all amino acids are indicated by their single- or three-letter codes; some examples are included below:</td>
</tr>
<tr>
<td></td>
<td>A, Ala, Alanine</td>
</tr>
<tr>
<td></td>
<td>C, Cys, Cysteine</td>
</tr>
<tr>
<td></td>
<td>D, Asp, Aspartate</td>
</tr>
<tr>
<td></td>
<td>E, Glu, Glutamic acid</td>
</tr>
<tr>
<td></td>
<td>F, Phe, Phenylalanine</td>
</tr>
<tr>
<td></td>
<td>G, Gly, Glycine</td>
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<tr>
<td></td>
<td>H, His, Histidine</td>
</tr>
<tr>
<td></td>
<td>I, Ile, Isoleucine</td>
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<tr>
<td></td>
<td>K, Lys, Lysine</td>
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<tr>
<td></td>
<td>N, Asn, Asparagine</td>
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<td>P, Pro, Proline</td>
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<td></td>
<td>Q, Gln, Glutamine</td>
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<tr>
<td></td>
<td>R, Arg, Arginine</td>
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<tr>
<td></td>
<td>S, Ser, Serine</td>
</tr>
<tr>
<td></td>
<td>T, Thr, Threonine</td>
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<tr>
<td></td>
<td>V, Val, Valine</td>
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<td></td>
<td>W, Trp, Tryptophan</td>
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<td></td>
<td>Y, Tyr, Tyrosine</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABP</td>
<td>Activity-based probe</td>
</tr>
<tr>
<td>ABRA</td>
<td>Activity-based probe ratiometric assay</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>B</td>
<td>Biotin</td>
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<tr>
<td>B1, B2</td>
<td>Bradykinin receptors 1 and 2</td>
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<td>Bio-PK</td>
<td>Activity-based probe with sequence: biotin-linker-Pro-Lys-diphenylphosphonate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSP</td>
<td>Brain serine protease</td>
</tr>
<tr>
<td>BSSP</td>
<td>Brain and skin serine protease</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>cDNA</td>
<td>Deoxyribonucleic acid; c is used to indicate the single-stranded complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DFP</td>
<td>Diflunisal phosphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ENMD-1068</td>
<td>(N^{\prime}-3)-methylbutyryl-(N^{\prime\prime}-6)-aminohexanoyl-piperazine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney cells</td>
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<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HSCTE</td>
<td>Human stratum corneum tryptic enzyme</td>
</tr>
<tr>
<td>kDa</td>
<td>Molecular weight in kilodalton</td>
</tr>
<tr>
<td>KLK</td>
<td>Human kallikrein-related peptidase protein</td>
</tr>
<tr>
<td>KLK1</td>
<td>Tissue kallikrein</td>
</tr>
<tr>
<td>KLKB1</td>
<td>Plasma kallikrein</td>
</tr>
<tr>
<td>KNRK</td>
<td>Kirsten virus-transformed normal rat kidney cells</td>
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<td>KNRKrPAR2</td>
<td>Rat PAR2-expressing KNRK cells</td>
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<td>KNRKrPAR2(R36A)</td>
<td>KNRK cells expressing a trypsin-resistant rat PAR2 mutant</td>
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<td>LEKTI</td>
<td>A serine proteinase inhibitor encoded by (\text{SPINK5}), which is associated with skin diseases</td>
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<tr>
<td>L-NAME</td>
<td>(N^{\omega})-nitro-L-arginine methyl ester, a nitric-oxide synthase inhibitor</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSP</td>
<td>Myelencephalon specific protein</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PA</td>
<td>Formerly known as SA; the clan is currently containing both serine and cysteine endopeptidases; the PA(S) subclan contains the serine proteinases of the S1 family</td>
</tr>
<tr>
<td>PAR</td>
<td>Proteinase-activated receptor of human (h), mouse (m) or rat (r) origin</td>
</tr>
<tr>
<td>PAR-AP</td>
<td>Proteinase-activated receptor-activating peptide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>QAR-AMC</td>
<td>(t)-butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>S1</td>
<td>Family of serine proteinases of clan PA (SA)</td>
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<td>Streptavidin</td>
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<td>siRNA</td>
<td>Silence RNA</td>
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<td>SPINK5</td>
<td>Gene encoding for LEKTI, a Kazal type-5 serine proteinase inhibitor</td>
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<td>STI</td>
<td>Soybean trypsin inhibitor</td>
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<td>TBST</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tissue growth factor type beta</td>
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<tr>
<td>TL</td>
<td>Tethered ligand of proteinase-activated receptors, which triggers their activation</td>
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<td>TRAP</td>
<td>Thrombin receptor-activating peptide</td>
</tr>
<tr>
<td>Trp</td>
<td>Trypsin</td>
</tr>
<tr>
<td>uPA (pro-uPA)</td>
<td>Urokinase plasminogen activator (proform of uPA)</td>
</tr>
<tr>
<td>VPR-AMC</td>
<td>t-butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

The major body of the introductory information and data, which are presented in this thesis, are included in the following six publications. Permission for reproduction of the content of these articles has been granted by the editorial office of the journals Biological Chemistry, Journal of Biological Chemistry, and Inflammation Research, and the respective co-authors.

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<thead>
<tr>
<th>Chapter</th>
<th>Publication</th>
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</table>
Chapter I.

Introduction
INTRODUCTION

1.1 Overview

Proteinases are long known for their roles in a variety of physiological and pathological processes. Amongst the different families of enzymes, the serine proteinases are gaining increasing popularity during the past decades. Members of this group include the vital enzymes of the digestive system, trypsin and chymotrypsin, as well as one of the major components of the coagulation system, thrombin. These enzymes exert their functions via different proteolytic pathways, including the activation of proteinase activated receptors (PARs), a G-protein-coupled family of cell surface receptors.

The 15-member family of the human kallikrein-related peptidases (KLKs) is a novel group of serine proteinases with either trypsin or chymotrypsin-like activity. The family is characterized by a remarkably wide, nevertheless distinct, tissue distribution and has been related to cancer. We hypothesized that the KLKs can participate in tumour signalling via PARs, similar to trypsin and thrombin. The following introductory sections will walk the reader through some general background information on serine proteinases and their mechanism of activation of proteinase-activated receptors. As well, an overview of the properties characterizing the tumour-derived KLKs and their clinical utility for cancer will be presented.

1.2 Serine Proteinases

1.2.1 General classification of proteinases

All living prokaryotic and eukaryotic organisms, from bacteria to mammals, as well as viruses, express proteolytic enzymes with similar or distinct functions (Travis et al., 1995; Klemba and Goldberg, 2002; Monod et al., 2002; Tong, 2002; Beers et al., 2000). These
enzymes can hydrolyse and cleave peptide bonds and are referred to as peptidases, proteases or proteinases. The term proteinase, instead of protease, is particularly popular to distinguish those enzymes showing preference against protein substrates from those, which exhibit preference against short peptides. Proteinases can target a polypeptide bond within or at the terminal sides of the substrate and as such are classified as endopeptidases or exopeptidases (Figure 1). Exopeptidases can be further subdivided into aminopeptidases and carboxypeptidases, depending on whether they cleave within the N-terminal or the C-terminal of the targeted substrate (Hooper, 2002).

According to Hartley, the endopeptidase group consists of: 1) serine, 2) cysteine, 3) aspartic, 4) metallo-peptidases (Hartley, 1960; Barrett et al., 2004). Later, three more groups were added to the above classes to complete the current classification, the threonine and glutamic peptidases, as well as a group of proteinases of unknown catalytic type (http://merops.sanger.ac.uk; Figure 1). Rawlings and Barrett used a different classification system to stratify proteinases (Rawlings and Barrett, 1993; Barrett et al., 2001; Rawlings et al., 2008). Their nomenclature uses the term peptidase unit to distinguish proteases by the nature of the active site and the term family to describe proteases that resemble in either their whole sequence or in the sequence that includes the catalytic site. They also use the term clan to include families of proteases that lack obvious sequence homologies but may show similarities at the linear order of the catalytic site, the tertiary structure or the specificity, and the sensitivity to inhibitors. Each family is given a letter corresponding to the catalytic type of the protease and a number that is assigned sequentially. Similarly a clan is identified by two letters, the first representing the catalytic type of the families that belong to the clan and the second letter being added in sequence. A clan that contains families of more than one type is identified with the letter P and the letter U is used when the catalytic type remains unknown.
According to Rawlings and Barrett, serine proteinases are classified into about 30 families and can be grouped into 6 main clans (Barrett and Rawlings, 1995). The serine proteinase family currently numbers 176 members, which represent about 30% of the total number of the known proteolytic enzymes (Puente et al., 2003; Yousef et al., 2003d; López-Otín and Matrisian, 2007). The majority of these proteinases are secreted enzymes (López-Otín and Matrisian, 2007). Chromosome 19 and 16 contain the two largest clusters of serine proteinases: the kallikrein family and the tryptase family, respectively, both belonging to the family S1, clan SA (currently known as clan PA) of serine proteinases along with chymotrypsin, trypsin, and elastase (Yousef et al., 2003d; Barrett et al., 2004; http://merops.sanger.ac.uk).
1.2.2 Catalytic activity of serine proteinases

The presence of the amino acid Ser within the enzyme’s active site characterizes all members of the serine proteinase group. In the majority of these enzymes, including the members of the S1 family, the three amino acids Ser, His, and Asp form the catalytic triad (Dodson and Wlodawer, 1998; Barrett et al., 2004). The amino acids of the triad are kept closely together to allow the formation of a hydrogen bond between the histidine and the serine residue, as well as between the histidine and the aspartate residue. These bonds are necessary to stabilize the cleft-like structure of the active site of the enzyme, where the polypeptide substrate can bind.

The mechanism of serine proteinase catalysis is referred to as limited proteolysis, since the proteinase selectively cleaves one or few peptide bonds within the target protein (Schultz and Liebman, 1997). The reaction is initiated when the carbonyl group of the target peptide bond (scissile bond) undergoes a nucleophilic attack by the hydroxyl group of the active Ser (Stryer, 1988; Warshel et al., 1989). This results in the formation of an acyl-enzyme intermediate of the proteinase complexed non-covalently with the substrate and the immediate release of a peptide with a free amino group. In the second step, a water molecule hydrolyses the acyl-enzyme intermediate to liberate the proteinase and a peptide with a free carboxyl group (Stryer, 1988; Warshel et al., 1989). An example of serine proteinase catalysis is given in Figure 2.
Figure 2: Mechanism of catalysis by serine proteinases like trypsin and chymotrypsin. **Acylation:** (A) A non-covalent proteinase-substrate complex is formed in the specificity pocket of the enzyme, orienting the scissile bond near the hydroxyl group of the active Ser\textsuperscript{195}. (B) Stabilized by Asp\textsuperscript{102}, His\textsuperscript{57} accepts a proton from Ser\textsuperscript{195}, allowing the nucleophilic oxygen atom of the hydroxyl group of Ser\textsuperscript{195} to attack the carbonyl carbon of the scissile peptide bond, forming a negatively charged tetrahedral intermediate. (C) and (D) The imidazole ring of His\textsuperscript{57} donates a hydrogen atom to the nitrogen of the scissile bond, allowing the release of a peptide with a free amino group (R1-NH\textsubscript{2}). The remainder of the substrate peptide is bound to Ser\textsuperscript{195} by an ester bond, forming an acyl-enzyme intermediate. **Deacylation:** (E) and (F) His\textsuperscript{57} attracts a hydrogen atom from a water molecule that diffuses in the active site, leaving an OH\textsuperscript{-} ion free to attack the carbonyl carbon that is bound to Ser\textsuperscript{195} and form a second tetrahedral intermediate. (G) A hydrogen atom is transferred from His\textsuperscript{57} to the oxygen atom of Ser\textsuperscript{195}, allowing breaking of the bond between Ser\textsuperscript{195} and the peptide. This facilitates the release of a peptide with a free carboxyl group (R2-COOH). The role of the Asp residue (contains a negatively charged oxygen ion), is to stabilize the positive form of His during both intermediate states, as well as to orient His appropriately in relation to Ser (not shown in Figure 2). Numbering of amino acids refers to the position in the polypeptide chain, according to chymotrypsin numbering (Hartley, 1964).
Schechter and Berger suggested that the amino acid residues of the polypeptide substrate from amino- to carboxy-terminal should be indicated as ...Pn, P2, P1 - P1', P2', Pn'... and their respective binding sub-sites within the proteinase as ...Sn, S2, S1 - S1', S2', Sn'... (Schechter and Berger, 1967). Proteolytic cleavage occurs between the P1 and P1' positions of the substrate. The S1 sub-site resides near the Ser residue in the active site and determines the substrate specificity of the serine proteinases (Hedstrom, 2002). In the case of family S1 of serine proteinases the specificity can be of trypsin, chymotrypsin or elastase-like type (Stryer, 1988; Hedstrom, 2002; Barrett et al., 2004; http://merops.sanger.ac.uk). The trypsin-like serine proteinases have Asp (or Glu) at their S1 site showing preference against basic amino acids, such as Arg or Lys, at P1 position. The chymotrypsin-like serine proteinases have a non-polar S1 sub-site and can accommodate aromatic or bulky non-polar amino acids, such as Trp, Phe or Tyr, within their active sites.

1.2.3 Regulation of serine proteinase activity

Despite the traditional belief that serine proteinases, like trypsin, are non-specific degradative enzymes that participate in protein catabolism, more recent evidence has shed light into more specific roles of serine proteinases in different settings. This proteolytic activity is maintained under tight control to achieve efficient catalysis in terms of space and time. One of the main regulators of proteolytic activity is the mechanism of zymogen activation and enzyme degradation. For example enterokinase acts on trypsinogen to form trypsin (Chen et al., 2003) and trypsin acts on chymotrypsinogen to form chymotrypsin (Gladner and Neurath, 1953; Dreyer and Neurath, 1955). Trypsin can additionally act as a negative regulator of its activity by self-coordinated cleavage (Kukor et al., 2003).
In addition, the equilibrium between the proteinases and their interacting inhibitors can determine the overall proteinase activity. Serine proteinase inhibitors can be categorized into non-canonical inhibitors, canonical inhibitors, and serpins, standing for serine proteinase inhibitors (Otlewski et al., 1999). The latter two types interact with their target proteinases in a substrate-like manner. The non-canonical inhibitors bind with their amino terminal to the proteinase forming a parallel β-sheet. Similar to their respective proteinases, serine proteinase inhibitors are also classified in families, which include the Kunitz-type inhibitors, the Kunitz-type soybean trypsin inhibitors (STI), the Kazal-type inhibitors, the hirudin family, and the serpins (Silverman et al., 2001; Laskowski et al., 2003).

Other factors that determine the enzyme localized activity include the specific localization of proteinases near the site of action (e.g. intracellular proteinases: Bond and Butler, 1987), the optimal pH conditions (Hashimoto and Yamamura, 1989; Kukor et al., 2003), and the effect of co-factors, such as calcium (Bode and Schwager, 1975; Chen et al., 2003; Kukor et al., 2003), ATP (Schmidt et al., 1999) or hormones (Ny et al., 1993).

1.2.4 Role of serine proteinases in physiology and pathophysiology

The tightly regulated proteinases or proteinase networks, as mentioned above, have an important role in many physiological and pathological processes. A few selected examples are the activation of the complement system (Reid and Porter, 1981), the blood coagulation cascade (Jackson and Nemerson, 1980), the fibrinolytic system (Francis and Marder, 1987), as well as the vital process of digestion (Rothman, 1977). As such, serine proteinases may be important for the processes performed by the immune and cardiovascular system. More recent evidence further suggests that serine proteinases may be responsible for skin desquamation (Suzuki et al., 1994; Caubet et al., 2004) and fertilization (Barros et al., 1996). A role in tumour invasion,
metastasis, and angiogenesis has also been suggested for mainly three classes of proteinases, the metallo, cysteine, and serine proteinases (Duffy, 1992; Pepper, 2001; Freije et al., 2003; Lah et al., 2006; López-Otín and Matrisian, 2007; Duffy et al., 2008). The urokinase plasminogen activator (uPA) is one such serine proteinase that has been implicated to several types of cancers (Duffy and Duggan, 2004). Of special interest is the effect of viral (Piedimonte et al., 1990; Barrett et al., 2004), bacterial (Dancer et al., 1990; Nickerson et al., 2007), and parasite (Breton et al., 1992; Maruo et al., 1991) proteinases interacting with the host immune system, which is the focus of current research. It is interesting to note that apart from the proteinase themselves, a deficiency in their inhibitors can also be a cause for diseases, such as rheumatoid arthritis (Griffiths et al., 1992; Hummel et al., 1997; Judex and Mueller, 2005), emphysema (Gadek, 1992), and the skin condition known as Netherton syndrome (Komatsu et al., 2008).

1.3 Proteinase Signalling via Proteinase-Activated Receptors (PARs)

A major feature of proteinases, which has gained popularity over the past decade, is their role in regulating cell signalling pathways that can determine cell fate and metabolism. In principle, a proteinase acting as an agonist on a cell surface receptor can participate in processes critical for the initiation and course of diseases ranging from asthma to cancer. In the following sections this unique role of proteinases will be introduced, aiming to convince the reader that proteinases can now be considered as hormone-bearing messengers with distinct rather than generalized targets.
1.3.1 Non-proteolytic pathways of proteinase signalling

Proteinases can regulate signalling processes via specific non-catalytic domains, which participate in protein-protein interactions outside of their active site (Hansen et al., 2008a). For example, thrombin proteolytic fragments have been shown to possess chemotactic and mitogenic properties. These peptides can interact with cell surface receptors, independently of proteolysis (Bar-Shavit et al., 1984; Bar-Shavit et al., 1986; Glenn et al., 1988). Another example is human lung β-tryptase, which, when rendered inactive by inhibitor treatment, can stimulate the replication of human airway-derived smooth muscle cells by an unknown mechanism, which it has been hypothesized to involve novel interactions but not any catalytic activity (Brown et al., 2002).

The non-catalytic properties of proteinases are commonly overlooked; therefore, a brief reference is included in this chapter to point out that the ability of proteinases to effect signalling via their non-catalytic domains is equally important to their catalytic properties. In the next sections we will focus on signalling by proteinases due to proteolysis.

1.3.2 Proteolytic pathways of proteinase signalling

Proteinases can proteolytically activate several components of many cell signalling pathways. Despite the norm that signalling requires direct involvement of a receptor, there are additional mechanisms via which a proteinase can indirectly mediate cell signalling. These direct and indirect mechanisms have been thoroughly studied in the settings of cancer and inflammatory diseases, and some examples will be summarized in the following paragraphs.

(a) Regulation of growth factor receptors. One of the earliest reported examples of proteolytic signalling came more than 3 decades ago, when the insulin-like actions of trypsin due to cleavage of insulin receptor were observed (Reiser & Rieser, 1964; Cuatrecasas, 1971;
Shoelson et al., 1988). One could expect that other growth factor signalling pathways may also be effected in a similar manner, but this hypothesis has yet to be examined.

(b) Regulation of agonists for growth factor and cytokine receptors. Proteinases can also activate receptors indirectly by generating receptor agonists/hormones from their pro-peptides, such as insulin from the proteolytic fragmentation of pro-insulin (Steiner et al., 1967; Hedo et al., 1983). Another example is the activation of the latent pro-form of TGF-β by plasmin (Annes et al., 2003). Furthermore, the activation of a G-protein-coupled receptor involved in membrane-localized matrix metalloproteinase (MMP) proteolysis, can result in the release from the cell surface of ‘heparin-binding EGF’ which can in turn activate epidermal growth factor (EGF) receptor (Prenzel et al., 1999). Apart from growth factor agonist release, the role of proteinases in regulating the release of various cytokines should not be overlooked. For instance, MMP17, a membrane-localized MMP, is able to cleave membrane-bound pro-TNF-alpha to generate active soluble TNF-alpha (English et al., 2000). Neutrophil proteinases can also regulate cytokine function, as in the case of proteinase-3, cathepsin-G and elastase, which mediate inactivation of IL-6, while proteinase 3 can activate the proform of tumour necrosis factor (TNF) and elastase and cathepsin-G can degrade TNF (Pham, 2006).

(c) Regulation of G-protein-coupled receptors. Proteinases can also initiate signalling via direct proteolysis of a G-protein-coupled receptor (GPCR) family, known as proteinase-activated receptors (PARs; Steinhoff et al., 2005; Hollenberg et al., 2008). The properties of this family will be detailed in the following sections. It is interesting to note that there are several cross-talk points between the different types of cell surface receptors, upon proteolytic activation. More specifically, trans-activation of EGF receptor can result from downstream signalling of a variety of GPCRs, such as proteinase-activated receptor 1 or PAR1 (Darmoul et al., 2004a,b; Bergmann et al., 2006).
(d) **Signalling targets that are not classical receptors.** In addition to membrane-localized receptors, a variety of other targets, which cannot be considered as classical receptors can also mediate signalling by proteinases. For instance, destruction of the extracellular matrix-integrin network can in principle effect the matrix-integrin-related signalling pathways. As an example, thrombin can activate metalloproteinases (Lafleur *et al.*, 2001), which can cause remodelling of the extracellular matrix and effect cell signalling. This mechanism is believed to involve the membrane type metalloproteinase-1 (MT1-MMP; Koshikawa *et al.*, 2005). Another novel proteinase-triggered signalling mechanism has been discovered for plasmin, which in addition to regulating PAR activity (Kuliopulos *et al.*, 1999) can cleave the annexin A2 heterotetramer, which is a matrix protein. Even though the details of the annexin A2-mediated signalling are still unknown, the generated signal can be chemotactic in human monocytes and monocyte-derived macrophages (Laumonnier *et al.*, 2006; Li *et al.*, 2007).

In summary, *via* direct targeting or an indirect ‘trans-activation’ process (activation of GPCRs or processing of agonists near or tethered to the cell surface), proteinases are able to regulate cell signalling. Furthermore, they can effect signalling by acting on the protein contents of the extracellular matrix and their interactions. This diversity of hormone-like signalling mechanisms of proteinase catalysis, which is summarized in Table 1, is exceeded only by the diversity of the proteinase families themselves.
Table 1. Diversity of proteolysis-dependent proteinase signalling.

<table>
<thead>
<tr>
<th>Proteinase action</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth factor receptor activation / processing</td>
<td>• Insulin receptor activation by trypsin (Cuatrecasas, 1971; Shoelson et al., 1988)</td>
</tr>
</tbody>
</table>
| Pre-pro-hormone / agonist precursor processing         | • Generation of Insulin from pro-insulin (Steiner et al., 1967; Hedo et al., 1983)  
|                                                      | • Activation of TGF-β by plasmin (Annes et al., 2003)                      
|                                                      | • Generation of TNF-α from Pro-TNF-α by mouse MMP17 (English et al., 2000)  
|                                                      | • Degradation of TNF by elastase and cathepsin-G and IL-6 by proteinase-3, cathepsin-G, and elastase (Pham, 2006). |
| Membrane-tethered agonist release                     | • ‘Heparin-binding EGF’ release by MMP (Prenzel et al., 1999)             |
| GPCR activation / dis-arming                          | • PAR activation or dis-arming (see section below)                        |
| Extracellular matrix and integrin cleavage            | • Thrombin activation of MMP which can lead to matrix remodeling (Lafleur et al., 2001; Koshikawa et al., 2005)  
|                                                      | • Plasmin can generate cell signalling by cleaving the annexin A2 heterotetramer (Laumonnier et al., 2006; Li et al., 2007) |

Adapted from Hansen et al., 2008a.

1.3.3 Proteinase-activated receptors

As alluded to the previous section, one of the most thoroughly studied targets of proteinases, are the family of proteinase-activated receptors, now known as PARs. Multiple events led to the historical discovery of this family. It was known for many years that thrombin, in addition to catalyzing the cleavage of fibrinogen to form fibrin clots, could regulate platelet function and trigger cellular mitogenesis (Chen and Buchanan, 1975; Di Cera, 2003), thereby implying a receptor-mediated process at the cell surface. In 1991, two different groups, using an expression cloning approach of cDNA libraries from thrombin target cells (a human megakaryocyte cell line; hamster lung fibroblasts) succeeded in identifying a member of the G-
protein-coupled receptor (GPCR) superfamily as a receptor for thrombin (Rasmussen et al., 1991; Vu et al., 1991). This receptor was originally termed the ‘thrombin receptor’ and was later named proteinase-activated receptor-1 or PAR₁, according to the revised nomenclature (Hollenberg and Compton, 2002).

The mechanism of PAR₁ activation involves the proteolytic unmasking of a cryptic receptor-tethered ligand sequence in its N-terminal domain (Vu et al., 1991; Coughlin, 2005). This sequence can bind to the cell surface loops of the receptor to trigger G-protein-coupled signalling (Figure 3, panel A). Synthetic peptides mimicking the tethered ligand can also activate PAR₁ in the absence of thrombin-mediated proteolysis (Vu et al., 1991; Figure 3, panel C, left portion). These synthetic receptor-activating peptides were originally named thrombin receptor activating peptides (TRAPs) and are now known as PAR-activating peptides (PAR-APs). The PAR-APs have proved to be key reagents to study the pharmacology of the PARs and their role in pathobiology, which has been extensively reviewed (Hollenberg et al., 2008; Bushell, 2007; Hansen et al., 2008a,b; Luo et al., 2007; Ossovskaya and Bunnett, 2004; Ramachandran and Hollenberg, 2008; Steinhoff et al., 2005).
**Figure 3: Activation and dis-arming of PARs.** Activation occurs by proteolytic unmasking of a receptor-tethered ligand (TL) sequence, which binds to its extracellular domain to trigger activation (lower portion of left panel). Alternatively (bottom panel, left portion), a synthetic peptide with a sequence representing the TL domain can activate the receptor without the need for proteolysis (bold arrow). As shown on the right, a proteinase that cleaves downstream of the TL sequence can ‘dis-arm’ the receptor (bold arrow), preventing activation by another enzyme, and can also cleave within the extracellular receptor domains (dashed arrow) to prevent the receptor from being stimulated by an activating peptide. The dis-armed receptor that retains its other extracellular domains can still respond to a PAR-activating peptide (bottom panel, right portion).
In the years following the discovery of PAR_1 several observations pointed out to the presence of other receptors that could respond to the PAR-APs. The proteinase-activated receptor (PAR) family currently numbers 4 members, the characteristics of which are summarized in Table 2.

PAR_2 was the next family member discovered during a search of a mouse liver-derived genomic library with probes for the bovine substance K receptor (Nystedt et al., 1994). This receptor was prone to activation by trypsin (but not thrombin) and, as in the case of PAR_1, could respond to a receptor-derived tethered ligand (TL) sequence, SLIGRL (Nystedt et al., 1994). Human PAR_2 was cloned shortly thereafter (Bohm et al., 1996). Triggered by the structure-activity relationship studies with PAR_2-APs using rat aorta and gastric smooth muscle preparations, a possible role of PAR_2 for regulating gastric and vascular function was suggested (Al-Ani et al., 1995).

The discovery of PARs 3 and 4 resulted from researching thrombin response in mice that were deficient either in PAR_1 (Ishihara et al., 1997) or PAR_3 (Kahn et al., 1998). The cloning of human PAR_4 was also performed from a lymphoma Daudi cell cDNA library (Xu et al., 1998). The mechanism of activation for PARs 2 and 4 to initiate cell signalling is similar to PAR_1, as is schematically presented in Figure 3. PAR_3 signalling mechanism remains an enigma, since it does not appear to signal on its own, but rather acts as a cofactor for thrombin-mediated activation of PAR_4 (Kahn et al., 1998). A possible regulation of PAR_1 signalling by heterodimerization with PAR_3 has also been suggested (McLaughlin et al., 2007).
Table 2: The PAR family. h stands for human, m for mouse, and r for rat receptor.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>PAR₁</th>
<th>PAR₂</th>
<th>PAR₃</th>
<th>PAR₄</th>
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<tbody>
<tr>
<td><strong>Amino acid composition</strong></td>
<td>425 aa (h)</td>
<td>397 aa (h)</td>
<td>374 aa (h)</td>
<td>385 aa (h)</td>
</tr>
<tr>
<td><strong>Tethered ligand sequence</strong></td>
<td>SFLLR (h)</td>
<td>SLIGKV (h)</td>
<td>TFRGAP (h)</td>
<td>GYPGQV (h)</td>
</tr>
<tr>
<td><strong>Selectively agonist peptide</strong></td>
<td>TFLLR-NH₂</td>
<td>SLIGRL-NH₂</td>
<td>Unknown</td>
<td>AYPGKF-NH₂</td>
</tr>
<tr>
<td><strong>Major activating proteinases</strong></td>
<td>Thrombin</td>
<td>Trypsin, Tryptase</td>
<td>Thrombin</td>
<td>Thrombin, Trypsin</td>
</tr>
<tr>
<td><strong>Major dis-arming proteinases</strong></td>
<td>Trypsin, Cathepsin G</td>
<td>Elastase, Chymase</td>
<td>Cathepsin G</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Adapted from Steinhoff et al., 2005.

1.3.4 Activation versus inhibition or dis-arming of PARs

In many target cells, multiple PARs are concomitantly expressed. For example, both of PARs 1 and 4 are expressed in human platelets and both PARs 1 and 2 are expressed in the vascular endothelium of many species (Coughlin, 2005). In addition, all PARs can be expressed in breast cancer cells (Kamath et al., 2001) and tissues of the central nervous system (Luo et al., 2007). In these settings serine proteinases can not only activate PARs, but also cleave downstream of their activation site, inhibiting any further activation from another proteinase (Figure 3, panel B, bold arrow). This process is called dis-arming. For example, in the context of lung inflammation or Pseudomonas infection, either neutrophil- or Pseudomonas-derived elastase can ‘dis-arm’ PAR₂, so as to render it resistant to trypsin activation (Dulon et al., 2003; 2005). However, a dis-armed PAR remaining at the cell surface with its other extracellular loops intact can still respond to a PAR-AP (Figure 3, panel C, right portion).

In addition, a serine proteinase can cleave within the extracellular loops of the receptor (e.g. within the second extracellular loop) (Figure 3, panel B, dashed arrows), which can cause
disturbance of its conformation and interfere with its signalling. This cleavage type is referred to as dis-abling and can render the receptor non-responsive to the PAR-APs, a key feature, which allowed the functional study of PARs.

It should be stressed that the ability of a proteinase to silence an individual PAR by ‘dis-arning’ or “dis-abling” may be as relevant physiologically as is the activation of a PAR. PARs can be activated or inhibited by multiple proteinases (Table 2); therefore PARs, unlike many other GPCRs, have multiple circulating proteinase ‘agonists’ and ‘antagonists’. For this reason, controlling PAR function by targeting the proteinases represents a challenging task.

1.3.5 Role of PARs in pathophysiology

Stemming from studies with both PAR-activating peptides and receptor antagonists, as well as PAR-null mice, it has become evident that the PARs, and, therefore, their activating/inactivating proteinases, play important roles in a number of pathophysiological settings. PAR activation can directly effect multiple systems, including the vascular, musculoskeletal, nervous (both central and peripheral), renal, respiratory, gastrointestinal tract, and can promote inflammation, pain, coagulation, neurodegeneration, as well as cancer metastasis and invasion (Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005; Bushell, 2007; Moffatt, 2007; Ramachandran and Hollenberg, 2008; Hansen et al., 2008a,b; Hollenberg et al., 2008). In the following paragraphs and Table 3, selected information on PAR effects is presented.

(a) Cardiovascular function. As mentioned above, thrombin can facilitate platelet aggregation via PAR1, which is important for thrombosis and haemostasis, two processes equally critical for the proper function of cardiovascular system or in cardiac surgery (Landis, 2007; Martorell et al., 2008). Peptide structure-activity studies with a rat vascular preparation
pointed to the presence of functional PAR2 in the endothelium (Al-Ani et al., 1995). With a bioassay utilizing isolated rat aorta tissue, it has been possible to document the ability of PAR2, as well as PAR1, to activate an endothelium-dependent, nitric oxide (NO)-mediated vascular relaxation (Muramatsu et al., 1992; Al-Ani et al., 1995; Saifeddine et al., 1996). In contrast, activation of vascular smooth muscle cells PAR1, but not PAR2, can cause vasoconstriction. The impact of PAR4 activation on vascular function is not yet clear, except that it appears to play a potential role in endothelium-leukocyte interactions (Vergnolle et al., 2002).

Another potential role for PAR2 in the context of cardiovascular disease may occur in the setting of ischemia-reperfusion where an increase in expression of PAR2 can promote vasodilatation (Napoli et al., 2000). An up-regulation of PAR2 in vessels with coronary atherosclerotic lesions has also been reported (Napoli et al., 2004). Furthermore, studies utilizing PAR1 or PAR2-deficient mice, have shown that PARs 1 and 2 can effect blood pressure and heart rate by causing hypotension (PARs 1 and 2) and tachycardia (PAR1) (Damiano et al., 1999).

(b) Inflammation. Based on the knowledge that mast cells can release a variety of proteinases at sites of inflammation, it was hypothesized that this could lead to activation of PARs. To study this hypothesis, the impact of PAR-APs on a rodent model of inflammation was compared to the inflammatory response of a known PAR2 activator, trypsin. In that model, small doses of a PAR2-AP or a PAR1-AP caused increased swelling and leukocyte infiltration, comparable to that caused by trypsin or thrombin (Vergnolle et al., 1999a,b). A PAR2-AP was also found to induce intestinal inflammation (Cenac et al., 2002). At the same time came the observation that PAR1 and PAR2, localized on neurons from the myenteric plexus of guinea-pig small intestine in primary culture, could respond to activation by PAR1-AP and PAR2-AP (Corvera et al., 1999). Inspired by these two sets of observations, the hypothesis that the
inflammatory response triggered by PARs 1 and 2 involves a neurogenic mechanism was further investigated (Steinhoff et al., 2000; de Garavilla et al., 2001). Hollenberg et al. (2004) also showed that the administration of a PAR4-AP caused the formation of oedema and leukocyte recruitment in a rat paw model of inflammation. However, this response was not dependent on a neurogenic mechanism (Hollenberg et al., 2004; Houle et al., 2005).

Subsequently, the relation of PAR2 activation and inflammation was investigated in the setting of arthritis (Ferrell et al., 2003). Notably, inhibition of PAR2 by a PAR2-targeted antibody or a PAR2 antagonist diminished inflammation in a murine arthritis model (Kelso et al., 2006).

(c) Nociception and neuronal cell signalling. All four PARs are widely distributed on neurons and their associated cells, such as astrocytes, both in the central and peripheral nervous system (Steinhoff et al., 2005; Luo et al., 2007). As mentioned above PARs 1 and 2, expressed in neuronal cells, are primarily responsible for triggering inflammation (Steinhoff et al., 2000; de Garavilla et al., 2001). It was further speculated that the feeling of pain (nociception) would also involve PAR activity on these cells (Vergnolle et al., 2001a,b; Asfaha et al., 2002; Vergnolle, 2004). However, apart from regulating the inflammatory response and nociception, neuronal PARs seem to play a more widespread physiological role. For example, it has been reported that PAR1 is up-regulated in the central nervous system in the setting of HIV encephalitis (Boven et al., 2003). In addition, PAR2 was shown to have a neuroprotective role in the setting of HIV infection (Noorbakhsh et al., 2005). Interestingly, in an animal model of multiple sclerosis the role of PAR2 was reversed (Noorbakhsh et al., 2006).

(d) Cancer and metastasis. Despite the fact that research has focused on the role of metalloproteinases in cancer (Freije et al., 2003; Folgueras et al., 2004), serine proteinases are also abundantly released from tumours and may contribute to the process of carcinogenesis (Stack et al., 1998; Camerer, 2007a). It has been suggested that the coagulation system in
general and thrombin specifically via PAR₁ may play an important role in tumour growth and metastasis (Nierodzik et al., 1998; Henrikson et al., 1999; Di Cera, 2003). Moreover, thrombin can also be a potent mitogen for tumour-derived, as well as normal cells, such as the chick embryo fibroblasts (Chen and Buchanan, 1975), presumably acting via PAR₁. This receptor was also shown to contribute to the migration of cultured breast tumour-derived cells through a reconstituted matrix or basement membrane (Even-Ram et al., 1998), whereas another study attributed this role to PARs 2 and 4, while showing that PAR₁ can have an opposite effect (Kamath et al., 2001). The ability of PAR₁ to play a role in cancer metastasis and invasion is underlined by the ability of tumour-derived matrix metalloproteinase-1 to activate the receptor and drive the process of migration and metastasis of breast carcinoma cells in a xenograft (Boire et al., 2005). A comparable role for PAR₂ in the setting of cancer would be expected (Shi et al., 2004). For instance, Darmoul et al. (2004a,b) has shown that both PARs 1 and 2 can promote proliferation of colon cancer cell lines downstream of EGFR trans-activation.
### Table 3. Potential (patho)physiological roles for PARs.

<table>
<thead>
<tr>
<th>PAR involved</th>
<th>Potential Role</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARs 1, 3, 4</td>
<td>Platelet activation, haemostasis</td>
<td>PARs regulate both secretion and aggregation; PARs 1 and 4 can play separate roles</td>
</tr>
<tr>
<td>PARs 1, 2, 4</td>
<td>Modulation of endothelial cell function.</td>
<td>Release of NO, von Willebrand factor; increased neutrophil adherence; cell migration</td>
</tr>
<tr>
<td>PARs 1, 2, 4</td>
<td>Modulation of vascular smooth muscle function</td>
<td>Activation of contractility; angiogenesis?</td>
</tr>
<tr>
<td>PARs 1, 2, 4</td>
<td>Inflammation</td>
<td>Role of PARs can be either pro- or anti-inflammatory and mediated via neurogenic (PARs 1 and 2) or non-neurogenic (PAR4) mechanisms</td>
</tr>
<tr>
<td>PAR2</td>
<td>Inflammatory response to joint injury</td>
<td>Key role for PAR2 in arthritis</td>
</tr>
<tr>
<td>PARs 1, 2, 4</td>
<td>Hyperalgesia and analgesia</td>
<td>PAR activation can increase (PAR2) or decrease (PAR1, PAR4) pain sensation</td>
</tr>
<tr>
<td>PARs 1, 2</td>
<td>Modulation of CNS neuronal and astrocyte function</td>
<td>Uregulation of PARs in the setting of CNS inflammation</td>
</tr>
<tr>
<td>PARs 1, 2</td>
<td>Promoting tumour cell growth and metastasis</td>
<td>Both PARs 1 and 2 may play roles, activated by tumour-derived serine proteinases (e.g. thrombin) and matrix metalloproteinases (e.g. MMP-1)</td>
</tr>
<tr>
<td>PARs 1, 2, 4</td>
<td>Modulation of intestinal function</td>
<td>Regulation of motility (smooth muscle), secretion (epithelial cell), and inflammation (enteric neuronal cells)</td>
</tr>
<tr>
<td>PARs 1, 2, 4</td>
<td>Modulation of renal vascular function</td>
<td>Regulation of flow and afferent arteriolar function</td>
</tr>
</tbody>
</table>

Adapted from Steinhoff et al., 2005; Hollenberg et al., 2008; Ramachandran and Hollenberg, 2008.

### 1.3.6 Proteinases and PARs as therapeutic targets

Given the wide range of pathophysiological applications for PARs, it is expected that PAR activation or inhibition would be critical for many pathological conditions, rendering PARs and their proteinases as attractive targets for drug development. Inhibiting proteinases themselves in a restricted environment may also have value in certain therapeutic settings. For instance, in a *Citrobacter rodentium* - murine infectious colitis model, serine proteinase-
dependent colonic inflammation was markedly decreased by the oral administration of the selective serine proteinase inhibitor, soybean trypsin inhibitor (STI; Hansen et al., 2005). However, because of: (a) the difficulty in synthesizing serine proteinase inhibitors that are enzyme-selective and (b) the great abundance of serine proteinases that can activate / inhibit PARs 1, 2, and 4, the proteinases themselves do not appear to be particularly attractive therapeutic targets in systemic approaches of blocking PAR activation. The focus has rather been on developing PAR inhibitors.

In particular, PAR₁ has been selected as an important therapeutic target, since blocking its activation could inhibit some of the adverse effects of thrombin, without effecting its participation in the coagulation cascade. The most promising PAR₁-targeted compounds, have already entered Phase II and Phase III studies (Camerer, 2007b; Schering-Plough Corp. news release, 2007), for evaluation of their use in clinical settings.

Despite the involvement in several pathological settings, PAR₂ has represented a considerable challenge in terms of developing a high potency receptor-selective antagonist. Thus far, a PAR₂ selective antagonist, ENMD-1068 (\(N^d\)-3-methylbutyryl-\(N^4\)-6-aminohexanoyl-piperazine), has proved of value in studies done in vivo, to reduce joint inflammation in a murine arthritis model (Kelso et al., 2006). While the synthesis of high potency orally available PAR₂ antagonists is still not possible, alternative approaches focus on developing PAR₂-targeted antibodies that block proteinase activation sites. For example, in the same arthritis model mentioned above, anti-PAR₂ antibodies (one monoclonal and one polyclonal) inhibited joint swelling (Kelso et al., 2006). That study further demonstrated the possibility of using siRNA to block activation of PAR₂, but neither the antibody nor the siRNA approach is sufficiently developed to be considered for therapeutic use as yet.
Inhibition of PAR₄ has also been attempted, both using peptide antagonists (Hollenberg and Saifeddine, 2001) and certain palmitoylated membrane-penetrating peptides, termed pepducins, which target the receptor intracellular loops (Covic et al., 2002a,b; Kuliopulos and Covic, 2003). However, both approaches have proved to be non-selective against PAR₄ (Covic et al., 2002b; Hollenberg et al., 2004), limiting in vivo applicability.

### 1.4 Kallikrein-related Peptidases (KLKs)

Even though thrombin and trypsin-like enzymes can be considered as strong agonists for PAR activation, the endogenous activators of PARs in many settings are still unknown. In an effort to identify the physiological regulators of PARs, we turned to a family of proteolytic enzymes, known as kallikrein-related peptidases or KLKs. In the following sections the attributes of these proteins within the serine proteinase class of enzymes will be summarized, in an effort to present the criteria that led us to consider KLKs as endogenous agonists of PARs in vivo.

#### 1.4.1 Historical Overview

Kallikreins are a group of secreted proteinases that belong to the clan SA of serine proteinases, currently known as clan PA, and to the family S1 (Barrett et al., 2004). The term kallikrein was introduced in the 1930s, referring to a protein secreted from pancreas, which in Greek is called the “Kallikreas” (good flesh) (Kraut et al., 1930; Werle, 1934), a name which could not foresee the mainly unfavourable role that kallikreins would serve in cancer. In the later years, two kallikreins were recognized: the plasma kallikrein (KLKB1) and the tissue (KLK1) kallikrein (Fiedler, 1979; Movat, 1979; Bhoola et al., 1992). These enzymes are the
products of two different genes, with low sequence or functional similarity. The tissue kallikrein enzyme has kininogenase activity and has been implicated in various physiological processes, including the control of blood pressure, electrolyte balance, and inflammation (Clements, 1989; Bhoola et al., 1992; Clements, 1997; Margolius, 1998a,b).

In the later years two other kallikrein genes, currently named \textit{KLK2} and \textit{KLK3}, encoding for the kallikrein proteins KLK2 (Schedlich et al., 1987) and KLK3 or PSA (prostate-specific antigen; Riegerman et al., 1989), were discovered. Currently the family consists of a total of 15 members, which encode for enzymes with trypsin or chymotrypsin-like activity (Yousef and Diamandis, 2001; Borgoño et al., 2004; Borgoño and Diamandis, 2004; Clements et al., 2004; Yousef et al., 2005). The number of the kallikrein isoforms may be even higher than these 15 members due to alternative splicing of kallikrein genes; a recent review has reported 82 different kallikrein gene transcript forms, including reference forms (Kurlender et al., 2005).

According to the recent nomenclature, kallikreins are now indicated as kallikrein-related peptidases, with the exception of KLK1, for which the older nomenclature has been retained (Lundwall et al., 2006). Their genes are symbolized as \textit{KLK} and the encoded proteins as KLK. In general, gene and protein numbers are assigned in sequence starting from the centromere to the telomere (Table 4).

Human kallikrein-related peptidase genes localize on chromosome 19q13.3-13.4 (Figure 4) and share a high degree of similarity with their chimpanzee (99%) and rodent (~77-85%) orthologues (Borgoño et al., 2004; Yousef et al., 2005), though it should be noted that the mouse and rat KLK families lack direct KLK2 and KLK3 orthologues. Within the human family, homology reaches the level of 30-50% at the gene and protein level, with the exception of KLKs 1-3 that show more striking similarities when compared to each other (80%) (Yousef and Diamandis, 2001; Borgoño et al., 2004).
Table 4: The kallikrein-related peptidases family; gene and protein nomenclature.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Other gene symbols</th>
<th>Protein</th>
<th>Other protein names</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK1</td>
<td>-</td>
<td>KLK1</td>
<td>Pancreatic/renal/urinary kallikrein</td>
</tr>
<tr>
<td>KLK2</td>
<td>-</td>
<td>KLK2</td>
<td>Human glandular kallikrein 1</td>
</tr>
<tr>
<td>KLK3</td>
<td>-</td>
<td>KLK3</td>
<td>Prostate-specific antigen (PSA)</td>
</tr>
<tr>
<td>KLK4</td>
<td>PRSS17, KLK-L1</td>
<td>KLK4</td>
<td>Prostase, KLK-L1 protein, EMSP1</td>
</tr>
<tr>
<td>KLK5</td>
<td>KLK-L2</td>
<td>KLK5</td>
<td>KLK-L2 protein, SCTE</td>
</tr>
<tr>
<td>KLK6</td>
<td>PRSS9</td>
<td>KLK6</td>
<td>Zyme, protease M, neurosin</td>
</tr>
<tr>
<td>KLK7</td>
<td>PRSS6</td>
<td>KLK7</td>
<td>SCCE</td>
</tr>
<tr>
<td>KLK8</td>
<td>PRSS19, TADG14</td>
<td>KLK8</td>
<td>Neuropsin, ovasin</td>
</tr>
<tr>
<td>KLK9</td>
<td>KLK-L3</td>
<td>KLK9</td>
<td>KLK-L3 protein</td>
</tr>
<tr>
<td>KLK10</td>
<td>PRSSL1</td>
<td>KLK10</td>
<td>NES1</td>
</tr>
<tr>
<td>KLK11</td>
<td>PRSS20</td>
<td>KLK11</td>
<td>TLSP, Hippostasin</td>
</tr>
<tr>
<td>KLK12</td>
<td>KLK-L5</td>
<td>KLK12</td>
<td>KLK-L5 protein</td>
</tr>
<tr>
<td>KLK13</td>
<td>KLK-L4</td>
<td>KLK13</td>
<td>KLK-L4 protein</td>
</tr>
<tr>
<td>KLK14</td>
<td>KLK-L6</td>
<td>KLK14</td>
<td>KLK-L6 protein</td>
</tr>
<tr>
<td>KLK15</td>
<td>HSRNASPH</td>
<td>KLK15</td>
<td>Prostin</td>
</tr>
</tbody>
</table>

Adapted from Yousef and Diamandis, 2001; Borgoño et al., 2004; Borgoño and Diamandis, 2004; Clements et al., 2004; Yousef et al., 2005.

1.4.2 Genes and proteins of kallikrein-related peptidases

The members of the KLK family share a high degree of sequence similarities with maximum homology in the proximity of the catalytic triad. The conserved regions WVLTAASHC, DLMLL, and GDSSGPL are flanking His57, Asp102, and Ser195 (chymotrypsin numbering; Hartley, 1964), respectively (Yousef and Diamandis, 2001; Clements et al., 2004). Similar to other serine proteinases, the KLKs have 10-12 conserved Cys residues that can form 5-6 disulfide bridges (Borgoño et al., 2004), with the exception of KLKs 8 (Yoshida et al., 1998) and 11 (Matsui et al., 2000), which can potentially link two monomers via an additional intermolecular Cys-Cys bridge. In addition, 25-30 invariant amino acids of serine proteinases, as
well as 39 amino acids that are completely conserved among the human KLKs, have also been identified (Yousef and Diamandis, 2001).

The molecular weight of the inactive proteins (hereafter referred to as proforms or zymogens) ranges from 23-26 kDa; however, most, if not all, kallikrein-related peptidases are glycoproteins in vivo, therefore exhibiting greater molecular weights (Borgoño et al., 2004). Most of the KLKs contain one or more putative N-glycosylation sites (Asn-X-Ser/Thr; X is any amino acid except Pro) and only a few kallikrein-related peptidases have potential O-glycosylation sites (Ser/Thr), amongst which only one has been reported (KLK1; Kellermann et al., 1988).

In terms of structure, the three classical kallikrein-related peptidases (KLKs 1-3) have a unique “kallikrein loop”, which may be responsible for the kininogenase activity of KLK1 (Yousef and Diamandis, 2001). This short sequence precedes the Asp in the active site of KLKs 1-3, but it is not present in its entirety in all of the other KLKs. Glycosylation of the “kallikrein loop”, as well as of other loops present in these enzymes, may be an important regulator of KLK expression and function. This hypothesis has been examined for the mouse KLK8, where glycosylation of the kallikrein loop determined the P2 specificity of the enzyme and assisted in the regulation of its secretion (Oka et al., 2002).
1.4.3 Enzymatic activity and KLK substrates

All KLKs are secreted as pre-pro-enzymes (Figure 4 and Borgoño and Diamandis, 2004; Borgoño et al., 2004); the only exception is a KLK4 splice variant, which has been predominantly localized in the nucleus (Korkmaz et al., 2001). In the extracellular space, KLKs are activated by virtue of a proteolytic excision of their pro-peptide, which allows the release of the mature enzyme (Borgoño and Diamandis, 2004; Borgoño et al., 2004). The length of the KLK pro-peptide varies from 4-9 amino acids, with the exception of KLK5, which has a 37-amino-acid pro-peptide, and the length of the mature protein ranges from 224-252 amino acids (Yousef and Diamandis, 2001; Borgoño and Diamandis, 2004; Borgoño et al., 2004). In all of the family members the S1 residue, which determines the enzymatic specificity, is located six amino acids upstream of the active Ser, at position 189 (according to chymotrypsin numbering; Hartley, 1964). The majority of KLKs possess trypsin-like activity, having either a Glu or an
Asp at the S1 region of the active site. In contrast, KLKs 3, 7, and 9 have either a Ser or Asn or Gly residue at their S1 site, which renders chymotryptic-like enzymatic activity (Borgoño et al., 2004).

Kallikrein-related peptidases must be functionally controlled to eliminate any chance of unwanted or unlimited proteolysis. Regulation of the KLK enzymatic activity is mainly achieved through a finely organized process of (auto)activation, (auto)degradation, and inhibition by serine proteinase inhibitors. At the first checkpoint, KLKs are secreted as inactive precursors and are activated upon cleavage close to the site where they exert their function. All kallikrein-related peptidases are cleaved after Arg or Lys, with the exception of KLK4 that is activated after Gln (Borgoño et al., 2004; Borgoño and Diamandis, 2004). This observation suggests that proteinases with trypsin-like activity, possibly the KLKs themselves, are putative activators of kallikrein-related peptidases. For example KLK2 (Lövgren et al., 1999; Denmeade et al., 2001) and KLK13 (Sotiropoulou et al., 2003) can auto-activate. As well KLK2 (Lövgren et al., 1997) and KLK15 (Takayama et al., 2001a) may activate pro-PSA, and KLK5 can activate pro-KLK7, participating in the skin desquamation cascade (Caubet et al., 2004; Brattsand and Egelrud, 1999). A recent study has also proposed a possible activation of pro-KLK5 by KLK14 within seminal plasma enzymatic cascade (Yoon et al., 2007; Emami and Diamandis, 2008).

The regulatory mechanism of KLK activity also involves deactivation of the proteins by internal cleavage, which can be self-mediated or derived by another proteinase (Borgoño et al., 2004; Borgoño and Diamandis, 2004). For example, autolytic degradation has been reported for KLK6 (Bernet et al., 2002; Magklara et al., 2003; Bayés et al., 2004) and KLK14 (Borgoño et al., 2004; Borgoño et al., 2007c). Furthermore, it has been suggested that the proteolytically inactivated fragments of KLK2 and KLK3 in seminal plasma are the products of action of a
proteinase with trypsin-like activity, potentially KLK2 (Lövgren et al., 1997; Christensson et al., 1990; Watt et al., 1986; Sensabaugh and Blake, 1990).

The third factor that exerts tight control on KLK activity is the interaction with inhibitors (Luo and Jiang, 2006; Borgoño et al., 2004; Borgoño and Diamandis, 2004). A selective KLK serpin inhibitor, named kallistatin, has been identified only for KLK1 (Chao et al., 1990; Zhou et al., 1992). In addition, it has been reported that inhibitory fragments of LEKTI (a Kazal-type inhibitor) may regulate the activity of several KLKs in the skin (Deraison et al., 2007; Borgoño et al., 2007b). In general, many kallikrein-related peptidases form complexes in vivo and/or in vitro with plasma inhibitors, primarily serpins, such as α1-antitrypsin, α1-antichymotrypsin and α2-antiplasmin, and the general proteinase inhibitor α2-macroglobulin (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Luo and Jiang, 2006). Restraint of KLK activity is also possible by allosteric inhibition by metal ions, such as zinc, as it has been reported for KLK5 and KLK7 (Debela et al., 2007a,b).

Several KLK substrates have been identified, including ECM proteins, fibronectin, laminin, the proform of urokinase plasminogen activator (pro-uPA), and plasminogen, growth factors, as well as myelin basic protein and amyloid precursor protein in the brain, and corneodesmosin in the skin (Borgoño et al., 2004, Borgoño and Diamandis, 2004; Figure 5). However, the majority of these studies have focused on in vitro analysis of the potential substrates, or have recruited methods using peptide-based libraries, such as phage display and combinatorial libraries (Felber et al., 2005; Borgoño et al., 2007a). As a consequence, the natural endogenous protein substrates for KLKs are yet to be discovered.
Figure 5: Potential KLK substrates. Examples are given for each suggested substrate group. MBP stands for Myelin Basic Protein, VIP for Vasoactive Intestinal Peptide, TGF-β for tissue growth factor type beta, and pro-uPA for proform of urokinase plasminogen activator. For details see Borgoño et al., 2004; Borgoño and Diamandis, 2004.

1.4.4 Tissue expression

One of the most remarkable assets of KLKs is that they share a wide range of expression in different tissues, as well as under physiological and pathological settings (Borgoño et al., 2004, Borgoño and Diamandis, 2004; Shaw and Diamandis, 2007). This characteristic co-expression of several members of the KLK family has given birth to several hypotheses on a common biological role or a possible functional collaboration of these serine proteinases (Brattsand et al., 2005; Pampalakis and Sotiropoulou, 2007; Yoon et al., 2007). It is interesting to note that expression of KLKs is under steroid hormonal regulation, particularly from estrogens, androgens, and progestins (Borgoño et al., 2004, Borgoño and Diamandis, 2004).
has recently been shown that hormonally regulated KLK expression may occur in a coordinated manner (Paliouras and Diamandis, 2007).

As an example of KLK co-expression, certain groups are expressed in the prostate (KLKs 2-4, 11, and 15), skin (KLKs 1, 4-11, 13, and 14), breast (KLKs 5, 6, 10, and 13), and the central nervous system (KLKs 6-9, 14) (Borgoño et al., 2004; Borgoño and Diamandis, 2004). Tissue-specific patterns of expression have also been documented for many alternative mRNA transcripts of KLKs. The isolation of kallikrein-related peptidases from biological fluids, such as serum, seminal plasma, and milk of lactating women, confirms that they are secreted in vivo (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Shaw and Diamandis, 2007). In situ hybridization and immunohistochemical studies have also detailed the tissue or cell type expression and intracellular localization of the inactive KLK proteins prior to secretion (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Petraki et al., 2006b).

The members of the kallikrein-related peptidase family are also abundantly expressed in groups in several types of cancers, especially hormone dependent malignancies (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Obiezu and Diamandis 2005). In addition many alternative KLK transcripts are differentially expressed in cancer and some are even cancer-specific. The specific role of KLKs in cancer will be thoroughly discussed in a later section.

1.4.5 Role in physiology and pathobiology

Role of tissue kallikrein. The biological role of KLK1 in inflammation has been thoroughly studied (Fiedler, 1979; Movat, 1979; Bhoola et al., 1992; Clements, 1997; Margolius, 1998a,b and Figure 5). Amongst the kallikrein-related peptidases, KLK1 has a high affinity for low molecular mass kininogen. Proteolysis of kininogen generates the decapeptide kallidin, which may then be converted to the nonapeptide bradykinin by other amino peptidases.
The physiological effects of kallidin and bradykinin are exerted through the B1 and B2 bradykinin receptors. This interaction plays a major role in regulation of blood pressure, vascular permeability, cell growth, electrolyte balance, as well as in inflammatory response. In addition, signaling through the G-protein-coupled bradykinin receptors can also cause oedema by increasing vascular permeability, smooth muscle spasm by stimulating contraction of non-vascular smooth muscle cells, and pain by stimulating C-fiber nociceptors. Finally, signaling through bradykinin receptors may contribute to innate immunity by stimulating the secretion of pro-inflammatory cytokines.

Semen liquefaction. A role for KLK2 (Deperthes et al., 1996), KLK3 (Lilja, 1985), and KLKs 5 and 14 (Michael et al., 2006; Emami and Diamandis, 2008; Emami et al., 2008), in seminal clot liquefaction after ejaculation has been suggested. In prostate secretion, the proteolytic activity of KLK3 and other kallikrein-related peptidases is obstructed by interaction with Zn$^{2+}$ ions. It has been shown that KLK3 inhibited by low concentrations of Zn$^{2+}$ can regain activity when semenogelin is added to chelate the Zn$^{2+}$ (Jonsson et al., 2005). After the ejaculatory mixing of prostate secretions with secretions from the seminal vesicles, Zn$^{2+}$ is rapidly chelated by semenogelins, resulting in release of active KLK3 (Lundwall and Brattsand, 2008). The enzyme in turn, fragments the semenogelins to allow seminal clot liquefaction, and release of spermatozoa, while the Zn$^{2+}$ remains free to control the activity of KLK3. A similar role has been suggested for KLK2 (Deperthes et al., 1996) and KLK5 (Michael et al., 2006), while KLK14 seems to have a role mainly as an activator of KLK3 within the seminal plasma cascade (Emami and Diamandis, 2008; Emami et al., 2008).

Skin desquamation. Three kallikrein-related peptidases, KLK5, KLK7, and KLK14, have been isolated in their active forms from the outermost layers of the stratum corneum (Brattsand and Egelrud, 1999; Hansson et al., 1994; Stefansson et al., 2006). Other KLKs are also
expressed in the epidermis (Komatsu et al., 2005; 2006b), however, their specific contribution to trypsin-like or chymotrypsin-like activity in the skin is still unknown. Several data are accumulating that propose the involvement of kallikrein-related peptidases in skin (patho)physiology. For example, a kallikrein-related peptidase cascade, involving KLKs 5 and 7, has been identified and suggested to effect skin desquamation via the degradation of intercellular (corneo)desmosomal adhesion molecules (Caubet et al., 2004; Brattsand et al., 2005). Moreover, KLK1, KLK6, KLK13, and KLK14 have also been suggested to be involved in this process due to their immunolocalization and ability to degrade desmoglein-1, one of the adhesive proteins in the corneodesmosome (Borgoño et al., 2007b). Notably, all the aforementioned KLKs can be regulated by LEKTI (Borgoño et al., 2007b), a Kazal-type inhibitor, genetic mutations of which can lead to skin pathological conditions, such as Netherton syndrome, where KLKs are also over-expressed (Chavanas et al., 2000; Komatsu et al., 2008).

Role of KLKs in the central nervous system. Even though little is know about the function of the other KLK family members, putative roles have been proposed based on site of expression, enzymatic specificity, and interaction with inhibitors. For example, amongst the KLKs, which are expressed in the central nervous system (CNS), KLK6 has been implicated in multiple sclerosis (Scairisbrick et al., 2002; Blaber et al., 2004), Alzheimer’s disease (Little et al., 1997; Diamandis et al., 2000b; Ogawa et al., 2000; Mitsui et al., 2002; Zarghooni et al., 2002), and Parkinson’s disease (Ogawa et al., 2000; Iwata et al., 2003). Furthermore, KLK8, also known as brain serine peptidase or neuropsin, may play a role in synaptic plasticity (Shimizu et al., 1998; Yousef et al., 2003c), long-term potentiation (Tamura et al., 2006), and neurodegeneration (Terayama et al., 2007). As a result of this information and the enzyme specificity of the KLKs, a cascade with functional role in the brain has been recently proposed (Yoon et al., 2007). Interestingly, several KLKs implicated in this cascade are highly expressed
by immune cells, such as T cells and macrophages, in settings of CNS inflammation (e.g. multiple sclerosis), and are under steroid hormone regulation (Scarisbrick et al., 2002; 2006).

1.4.6 KLK expression in cancer

Kallikrein-related peptidases are expressed in several types of cancer, mainly hormone dependent, such as breast, ovarian, and prostate (Diamandis et al., 2000a; Borgoño and Diamandis, 2004; Borgoño et al., 2004; Obiezu and Diamandis 2005; Yousef et al., 2005). Further, as mentioned above, many alternative KLK transcripts are differentially expressed in cancer. For example, a KLK5 and a KLK7 variant are highly expressed in ovarian cancer cell lines, compared with normal ovarian epithelial cells (Dong et al., 2003).

PSA/KLK3 has served as a valuable biomarker for monitoring prostate cancer and has also been evaluated as a marker for diagnosis and prognosis of the disease (Loeb and Catalona, 2007). This method can reach up to 75% sensitivity and specificity, when the ratio of free-to-total PSA is used along with KLK2 (Becker et al., 2001; Catalona et al., 1998). Apart from KLKs 2 and 3, KLK11 is a novel promising biomarker for the detection of prostate cancer. Recent work utilizing PSA, KLK2 and KLK11 in a prostate cancer neural network resulted in clinically higher levels of tumour detection (Stephan et al., 2005; 2006). The clinical applicability of other members of the KLK family for the detection or prognosis of prostate, breast, and ovarian cancer, which has been under investigation for the past decade, is summarized in Table 5.

In breast cancer patients KLKs 5 and 14 are the most promising biomarkers with clinical applicability for disease diagnosis and prognosis (Table 5). In ovarian carcinoma a panel of kallikrein-related peptidases, such as KLKs 5, 6, and 10, are up-regulated in the serum of patients or in cell lines at the mRNA and/or the protein level (Table 5). Recently, a study on
KLK11 expression in serum proved the clinical value of this proteinase in distinguishing ovarian cancer cases from healthy controls, as well as malignant from benign cases with high levels of cancer antigen 125 (CA125) (McIntosh et al., 2007). Moreover KLKs 5, 10, and 14 may possess clinical value for testicular cancer prognosis, as well as KLK13 may aid in diagnosis of this type of cancer (Table 6). Up-regulation of KLKs 6 and 10 has also been identified in uterine serous papillary tumours, an aggressive type of endometrial cancer, and KLKs 7 and 8 have been related to cervical cancer, one of the most common gynaecological cancers (Table 6).

In addition to hormone-related malignancies, KLK expression has been related to several other types of tumours (Table 6). For example KLK11 is over-expressed in a specific subgroup of neuroendocrine lung carcinomas, KLKs 6 and 10 are over-expressed in pancreatic and colon cancer, and KLK8 in colon cancer, as well as KLK10 is down-regulated in acute lymphoblastic leukemia. Cases of lung, skin, salivary gland, renal, brain, and head and neck carcinomas have also been reported, where dys-regulated expression of kallikrein-related peptidases has been associated to cell malignancy (Table 6).

It has been suggested that a multiparametric analysis of kallikrein-related peptidases and other markers, such as CA125, rather than the use of one marker alone, may result in the highest possible clinical specificity and sensitivity for cancer diagnosis and prognosis (Borgoño et al., 2004). Our group has recently showed that combinations of eight KLKs can achieve areas under the ROC curve of 0.994 in separating ovarian cancer from benign tumours and 0.961 in separating ovarian from other cancer groups. Among the protein panel, KLKs 6, 7, 8, and 10 were the most specific for differential diagnosis of ovarian carcinoma from benign diseases and other cancer types (Shih et al., 2007). Another proof of the importance of a multiparametric analysis to detect cancer in patients, where conditional markers fail, is the finding that in 100% of ovarian tumours not expressing CA125, the markers KLKs 6 and 10, as well as osteopontin
(OPN) and claudin 3 were present (Rosen et al., 2005). These findings not only support the idea of co-expression and functional collaboration of KLKs, but also point to the significance of a KLK network for the tumour micro-environment. As a proof of principle, an in vivo analysis of cancer cells expressing KLKs 4, 5, 6, and 7, showed 92% mean increase in tumour burden when cells were inoculated into the peritoneum of nude mice (Prezas et al., 2006a).

Table 5: Human KLKs as biomarkers of prostate, breast, and ovarian cancer.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Gene or Protein</th>
<th>Clinical application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>KLK2</td>
<td>Diagnosis, prognosis, and monitoring</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK3/PSA</td>
<td>Diagnosis, prognosis, and monitoring</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK5</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK10</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK11</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK11</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK14</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Prostate</td>
<td>KLK15</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK3/PSA</td>
<td>Diagnosis, favourable prognosis, resistance to therapy</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK5</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK5</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK7</td>
<td>Unfavourable prognosis (Talieri et al., 2004)</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK7 (full length variant)</td>
<td>Favourable prognosis (Holzscheiter et al., 2006)</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK9</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK10</td>
<td>Resistance to therapy</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK10</td>
<td>Predictive of invasiveness</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK13</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK14</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK14/KLK14</td>
<td>Diagnosis (Fritzsche, 2006)</td>
</tr>
<tr>
<td>Breast</td>
<td>KLK15</td>
<td>Favourable prognosis</td>
</tr>
</tbody>
</table>

1KLK = kallikrein-related peptidase gene; KLK = kallikrein-related peptidase protein.
2Adapted from Borgoño et al., 2004; Borgoño and Diamandis, 2004; Obiezu and Diamandis, 2005; Yousef et al., 2005; additional references are stated in parenthesis.
Table 5 (continued)

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Gene or Protein(^1)</th>
<th>Clinical application(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian</td>
<td>(KLK4)</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK4)</td>
<td>Resistance to therapy (Xi et al., 2004)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK5)</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK6/)(KLK6)</td>
<td>Unfavourable prognosis and diagnosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK6)</td>
<td>Diagnosis and monitoring</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK7/)(KLK7)</td>
<td>Unfavourable prognosis (Shan et al., 2006)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK8/)(KLK8)</td>
<td>Favourable prognosis (Borgoño et al., 2006)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK8)</td>
<td>Diagnosis and monitoring</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK9)</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK10)</td>
<td>Unfavourable prognosis, diagnosis, and monitoring</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK10)</td>
<td>Unfavourable prognosis and diagnosis (Shvartsman et al., 2003)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK11)</td>
<td>Favourable prognosis and diagnosis (McIntosh et al., 2007)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK11)</td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK13)</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK14)</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK14)</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>Ovarian</td>
<td>(KLK15)</td>
<td>Unfavourable prognosis</td>
</tr>
</tbody>
</table>

\(^1\)KLK = kallikrein-related peptidase gene; KLK = kallikrein-related peptidase protein.
\(^2\)Adapted from Borgoño et al., 2004; Borgoño and Diamandis, 2004; Obiezu and Diamandis, 2005; Yousef et al., 2005; Bignotti et al., 2006; additional references are stated in parenthesis.
Table 6: Human KLKs as biomarkers of other types of cancer.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Gene or Protein</th>
<th>Expression and / or clinical application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular</td>
<td><em>KLK5</em></td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Testicular</td>
<td><em>KLK10</em></td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Testicular</td>
<td><em>KLK13</em></td>
<td>Diagnosis (5 splice variants expressed only in normal testis; Chang et al., 2001)</td>
</tr>
<tr>
<td>Testicular</td>
<td><em>KLK14</em></td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Uterine</td>
<td><em>KLK6</em></td>
<td>Monitoring and prediction to therapy (Santin et al., 2005)</td>
</tr>
<tr>
<td>Uterine</td>
<td><em>KLK8/KLK8</em></td>
<td>Up-regulation (Jin et al., 2006)</td>
</tr>
<tr>
<td>Uterine</td>
<td><em>KLK10</em></td>
<td>Diagnosis (Santin et al., 2006)</td>
</tr>
<tr>
<td>Cervical</td>
<td><em>KLK7/KLK7</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Cervical</td>
<td><em>KLK8/KLK8</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Brain</td>
<td><em>KLK6</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Brain</td>
<td><em>KLK7</em></td>
<td>Unfavourable prognosis (Prezas et al., 2006b)</td>
</tr>
<tr>
<td>Head and neck SCC</td>
<td><em>KLK10</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Non-small cell lung AC</td>
<td><em>KLK5</em></td>
<td>Up-regulation (Planque et al., 2005)</td>
</tr>
<tr>
<td>Non-small cell lung AC</td>
<td><em>KLK7</em></td>
<td>Down-regulation (Planque et al., 2005)</td>
</tr>
<tr>
<td>Non-small cell lung AC</td>
<td><em>KLK8</em></td>
<td>Favourable prognosis (Sher et al., 2006)</td>
</tr>
<tr>
<td>Non-small cell lung AC</td>
<td><em>KLK10/KLK11</em></td>
<td>Up-regulation (Planque et al., 2006)</td>
</tr>
<tr>
<td>Neuroendocrine C2 lung AC</td>
<td><em>KLK11</em></td>
<td>Unfavourable prognosis</td>
</tr>
<tr>
<td>Non-small cell lung AC</td>
<td><em>KLK13/KLK14</em></td>
<td>Up-regulation (Planque et al., 2008)</td>
</tr>
<tr>
<td>Salivary gland</td>
<td><em>KLK6</em></td>
<td>Down-regulation (Darling et al., 2006a)</td>
</tr>
<tr>
<td>Salivary gland</td>
<td><em>KLK13</em></td>
<td>Up-regulation (Darling et al., 2006b)</td>
</tr>
<tr>
<td>Skin SCC</td>
<td><em>KLK8</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Skin SCC, melanoma</td>
<td><em>KLK6/KLK6</em></td>
<td>Up-regulation (Klucky et al., 2007)</td>
</tr>
<tr>
<td>Colon</td>
<td><em>KLK1</em></td>
<td>Down-regulation</td>
</tr>
<tr>
<td>Colon</td>
<td><em>KLK6</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Colon</td>
<td><em>KLK8</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Colon</td>
<td><em>KLK10</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>RCC</td>
<td><em>KLK6</em></td>
<td>Unfavourable prognosis (Petraki et al., 2006a)</td>
</tr>
<tr>
<td>Pancreas</td>
<td><em>KLK6</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Pancreas</td>
<td><em>KLK10</em></td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Leukemia</td>
<td><em>KLK10</em></td>
<td>Down-regulation due to hyper-methylation</td>
</tr>
</tbody>
</table>

1AC = adenocarcinoma; SCC = squamous cell carcinoma; RCC = renal cell carcinoma.
2KLK = kallikrein-related peptidase gene; KLK = kallikrein-related peptidase protein.
3Adapted from Borgoño et al., 2004; Borgoño and Diamandis, 2004; Obiezu and Diamandis, 2005; Yousef et al., 2005; additional references are stated in parenthesis.
1.4.7 Role of kallikrein-related peptidases in carcinogenesis

The mechanism via which KLKs in vivo may promote or inhibit carcinogenesis is as yet unknown. As already mentioned in section 1.4.3 and Figure 5, several KLKs, similar to matrix metalloproteinases, can cleave ECM proteins or proteins associated with tissue remodelling, cell growth, adhesion, and migration (Borgoño et al., 2004; Borgoño and Diamandis, 2004). This underlines a possible role for the members of the KLK family in angiogenesis and tumour development, as well as in invasion and metastasis. KLKs can also activate proteins with known tumourigenicity; for example KLK2 (Frenette et al., 1997) and KLK4 (Takayama et al., 2001b) are known to activate the pro-form of urokinase plasminogen activator (uPA; Duffy and Duggan, 2004), a serine proteinase, which converts plasminogen to plasmin. Plasmin can subsequently degrade ECM proteins and activate members of the matrix metalloproteinase family, thereby facilitating tumour invasion and metastasis. In our study we investigated the hypothesis that kallikrein-related peptidases can interact with proteinase-activated receptors, which are also highly expressed and activated in a variety of tumours (Steinhoff et al., 2005).

In summary, kallikrein-related peptidases may be able to facilitate cleavage of the extracellular matrix proteins, activation of hormones, growth factors, other proteinases or receptors, to facilitate angiogenesis, tumour growth, invasion, and metastasis (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Hansen et al., 2008b). We anticipate that the KLKs, their targets or their potential receptors will in the future aid in the development of effective cancer therapeutic approaches.
1.5 Rationale, Hypothesis, and Objectives

1.5.1 Rationale

Proteinase activated receptors (PARs) are a family of G-protein-coupled cell surface receptors that are activated by proteolytic removal of their extracellular N-terminus. The family consists of 4 members, of which PARs 1, 3, and 4 are mainly cleaved by thrombin, while PAR2 is cleaved by trypsin-like proteinases, such as trypsin and proteinases of the coagulation cascade (Steinhoff et al., 2005). Localization studies have revealed an abundant expression of PARs in many tissues, including tumour sites. They have also been associated with nociception and inflammation, having either an anti- or pro-inflammatory role in different pathological settings, such as multiple sclerosis and HIV infection. Despite the wide range of applications for these receptors, their endogenous agonists in settings where trypsin or thrombin are absent are still unknown.

Kallikrein-related peptidases (KLKs) belong to a novel family of 15 secreted serine proteinases (Borgoño et al., 2004; Borgoño and Diamandis, 2004). The majority of KLKs have trypsin-like activity and only three members of the family exhibit chymotrypsin-like activity. The most remarkable characteristic of the family is their wide tissue distribution and their coordinated expression. Certain KLKs are expressed in different tissues and have been related to several pathological conditions. Furthermore, the majority of KLKs are abundantly expressed in cancer and in tissues and cells where PARs have well-recognized roles. Therefore, there has been extensive research on the clinical value of KLKs for the purpose of cancer diagnosis and prognosis. Even though in vitro and bioinformatics mining for KLK substrates has identified targets, such as growth factors, the urokinase plasminogen activator, and matrix proteins, we
believe that these substrates can only explain part of the role of KLKs in cancer. Thus, the potential substrates responsible for the \textit{in vivo} function of KLKs are yet to be found.

1.5.2 Hypothesis

The central hypothesis is that kallikrein-related peptidases can exert their (patho)physiological effects by targeting proteinase-activated receptors and their respective signalling pathways. To test this hypothesis, it was necessary to produce recombinant active KLKs to investigate their impact on the function of proteinase-activated receptors (PARs) \textit{in vivo}. We focused on KLKs 5, 6, and 14, as representative KLKs with comparable trypsin-like activity, wide tissue distribution, and abundant expression in several types of cancer. The objectives of this work are summarized as follows.

1.5.3 Objectives

1. Production and purification of active KLK5, KLK6, and KLK14.
2. Investigation of the interaction of KLK5, KLK6, and KLK14 with PARs using:
   a. PAR synthetic peptides
   b. Cell cultured model systems expressing PARs 1, 2, 4, and isolated human (PARs 1 and 4) and rat platelets (PAR$_4$)
   c. Platelet and vascular tissue bioassays.
3. Confirmation of PARs as KLK cell surface targets \textit{ex vivo} and \textit{in vivo} with:
   a. Cell surface receptor-targeted proteomics
   b. Mouse models of inflammation.
4. Develop a method to define the physiologically relevant concentrations of active KLKs in cancer-derived biological fluids.
Chapter II.

Production, Purification, and Activation of KLK5, KLK6, and KLK14

ORIGINAL PUBLICATION:
PRODUCTION, PURIFICATION, AND ACTIVATION OF KLK5, KLK6, AND KLK14

2.1 Introduction

As outlined in the objectives, the first goal was the production of recombinant active KLKs. To investigate our hypothesis we chose three representative members of the human kallikrein-related peptidase family. Since activation of PARs is mediated by trypsin-like proteolysis, we selected the tryptic kallikrein-related peptidases 5, 6, and 14. These KLKs are characterized by high catalytic properties and their expression has been related to several pathological and physiological conditions. In the following sections some background information regarding these three enzymes is summarized (Table 7 and Figure 6). This chapter further describes the production of active KLKs 5, 6, and 14 utilized to investigate our central hypothesis.

2.1.1 Kallikrein-related peptidase 5

Kallikrein-related peptidase 5 gene (KLK5) has been previously cloned by two different groups, given the names kallikrein-like gene-2 (KLK-L2) (Yousef and Diamandis, 1999) and human stratum corneum tryptic enzyme (HSCTE; Brattsand and Egelrud, 1999). The protein, now known as KLK5, is produced as a pre-pro-enzyme, which consists of a 29-amino acid signal peptide, followed by a 37-amino acid activation peptide. Mature KLK5 exhibits trypsin-like activity and its predicted molecular mass is 25 kDa (Yousef and Diamandis, 1999; Brattsand and Egelrud, 1999). In recombinant yeast preparations, four heavily glycosylated forms of molecular masses of about 44, 40, 35, and 30 kDa have been identified (Michael et al., 2005).
Activation of the enzyme requires cleavage after an arginine residue by a trypsin-like serine proteinase (Brattsand and Egelrud, 1999). Even though it has been reported that KLK5 can auto-activate (Brattsand et al., 2005), there has also been evidence against this hypothesis suggesting that another enzyme may activate KLK5 (Michael et al., 2005). However, this activating proteinase has not yet been identified in vivo. Regulation of KLK5 enzyme activity is achieved via interactions with plasma inhibitors, such as α2-antiplasmin and antithrombin (Luo and Jiang, 2006; Michael et al., 2005), the Kazal-type inhibitor LEKTI (also known by the gene name SPINK5; Borgoño et al., 2007b), as well as by zinc ions (Michael et al., 2006; Debela et al., 2007a). Fragmentation by KLK5 of several extracellular matrix components, such as collagens type I, II, III, and IV, fibronectin and laminin, has been confirmed in vitro. Finally, a role for KLK5 in angiogenesis has been suggested due to the release of an angioptatin isoform from plasminogen (Michael et al., 2005).

KLK5 has been shown to be estrogen/progestin-regulated (Yousef and Diamandis, 1999) and highly expressed in endocrine or hormone-responsive tissues such as testis, ovary, breast, and skin (Brattsand and Egelrud, 1999; Yousef and Diamandis, 1999; Borgoño et al., 2004; Borgoño and Diamandis, 2004). Recent studies have shown that KLK5 is differentially regulated in a variety of hormone-dependent malignancies, including ovarian (Kim et al., 2001), breast (Yousef et al., 2002d), prostate (Yousef et al., 2002c), and testicular (Yousef et al., 2002b) cancer. Moreover, the protein may be a potential biomarker for breast and ovarian cancer (Diamandis et al., 2003; Yousef et al., 2003e). In addition, KLK5 has been suggested to have an important role in skin desquamation (Brattsand and Egelrud, 1999; Ekholm et al., 2000; Caubet et al., 2004; Brattsand et al., 2005; Borgoño et al., 2007b) and skin physiology (Komatsu et al., 2003). As well, it may be involved in seminal clot liquefaction and in prostate cancer progression, as part of a greater KLK cascade (Michael et al., 2006). Even though the
activation process for KLK5 has not been studied in detail, a potential cross-activation between KLK5 and KLK14 or other members of the KLK family has been suggested (Yoon et al., 2007). A recent study has also indicated that KLK14 may be able to activate KLK5 in vitro and suggested the involvement of these KLKs in an enzyme cascade which is active in skin and seminal plasma (Emami and Diamandis, 2008).

2.1.2 Kallikrein-related peptidase 6

The KLK6 gene was originally cloned by three different groups from breast tissue (Anisowicz et al., 1996), brain (Little et al., 1997), and from a colon carcinoma cell line (Yamashiro et al., 1997). Not known at that time to represent the same enzyme, these proteinases were given the names protease M, zyme, and neurosin, respectively. The rat orthologue of KLK6 is known as myelencephalon-specific protease (MSP; Scarisbrick et al., 1997; Blaber et al., 2002; Scarisbrick et al., 2002). This protein has been reported to have enzymatic properties similar to human KLK6 (Blaber et al., 2002; Bernett et al., 2002). Mouse ortho-loques of human KLK6 have also been identified as brain and skin protease (BSSP) (Meier et al., 1999) and brain serine protease (BSP) (Matsui et al., 2000).

The regulation of KLK6 activity is mainly achieved due to the mechanism of activation and inactivation of the protein. Cleavage after the Lys\textsuperscript{15} of a 5-amino acid activation peptide of KLK6 (Glu-Glu-Gln-Asn-Lys) releases the mature, active enzyme (Gomis-Ruth et al., 2002). It has been reported that KLK6 can be auto-catalytically activated (intrinsic activation) (Little et al., 1997; Magklara et al., 2003; Bayés et al., 2004) via a two-step process in which an internal bond in the activation peptide is recognized and cleaved first, followed by the removal of a dipeptide to yield an active enzyme (Bayés et al., 2004). However, the preference of KLK6-mediated cleavage towards Arg versus Lys residues, led Bernett et al.
(2002) to suggest that a distinct Lys-specific proteinase is activating pro-KLK6 \textit{in vivo} (extrinsic activation). The preference for basic amino acids at P1 position was later verified by our group and others (Magklara \textit{et al.}, 2003; Angelo \textit{et al.}, 2006; Li \textit{et al.}, 2008; Sharma \textit{et al.}, 2008). In addition KLK6 can auto-degrade resulting in structural modifications of the protein and loss of its enzyme activity (Bernett \textit{et al.}, 2002; Magklara \textit{et al.}, 2003; Bayés \textit{et al.}, 2004). The main cleavage site of this inactivation is on residue Arg^76, which is the most solvent-accessible arginine residue in the structure (Bernett \textit{et al.}, 2002). It has recently been shown that the rate of auto-activation is slower than the rate of auto-degradation (Blaber \textit{et al.} 2007).

A few studies have been done in an attempt to determine the physiological substrates and inhibitors of KLK6. For example, it has been reported that rat myelin basic protein (MBP) is extensively and rapidly degraded by KLK6 \textit{in vitro} (Bernett \textit{et al.}, 2002). In addition, Little \textit{et al.} (1997) were able to detect amyloidogenic fragments when KLK6 was co-expressed with amyloid precursor protein (APP) in HEK-293 cells. Finally, our group has previously shown that KLK6 can \textit{in vitro} degrade APP (Magklara \textit{et al.}, 2003), as well as components of the extracellular matrix (ECM), such as plasminogen, fibrinogen, fibronectin, vitronectin, collagen type I, II, III, and IV, and laminin (Magklara \textit{et al.}, 2003; Borgoño and Diamandis 2004; Ghosh \textit{et al.}, 2004). KLK6 can also form complexes with the proteinase inhibitors \(\alpha_2\)-antiplasmin and antithrombin III (Magklara \textit{et al.}, 2003), and a complex of KLK6 with \(\alpha_1\)-antichymotrypsin in milk and ascites fluid of ovarian cancer patients has been described (Hutchinson \textit{et al.}, 2003). Recently, LEKTI has been suggested as a potential inhibitor of KLK6 in skin desquamation (Borgoño \textit{et al.}, 2007b).

KLK6 expression is regulated by hormones (mainly estrogens and progestins) and the protein seems to be widely expressed in human tissues (Shaw and Diamandis 2007; Yousef \textit{et al.}, 1999). The secreted protein is found in cerebrospinal fluid (CSF; Diamandis \textit{et al.}, 2000d;
Okui et al., 2001), vaginal fluid (Shaw et al., 2007), breast milk and breast cyst fluid (Diamandis et al., 2000d), nipple aspirate fluid (Diamandis et al., 2000d; Sauter et al., 2004), and ovarian cancer ascites (Shaw and Diamandis 2007; Luo et al., 2006). As well, the enzyme seems to be a clinically useful biomarker for hormonally-regulated cancers, such as breast and ovarian (Borgoño et al., 2004; Borgoño and Diamandis 2004; Yousef et al., 2005). Furthermore, studies of tissues from patients with multiple sclerosis have localized KLK6 at the sites of myelin degradation (Scarisbrick et al., 2002), pointing to a role in neurodegenerative diseases (Blaber et al., 2004). KLK6 is also differentially regulated in Alzheimer’s (Diamandis et al., 2000b; Ogawa et al., 2000; Shimizu-Okabe et al., 2001; Mitsui et al., 2002; Zarghooni et al., 2002) and Parkinson’s disease (Ogawa et al., 2000, Iwata et al., 2003).

2.1.3 Kallikrein-related peptidase 14

The gene for kallikrein-related peptidase 14 (KLK14) has been previously identified by positional candidate cloning (Hooper et al., 2001; Yousef et al., 2001). KLK14 is synthesized as an inactive precursor, containing a 6-amino acid N-terminal pro-peptide (Yousef et al., 2001; Hooper et al., 2001). Proteolytic removal of the pro-peptide by cleavage between residues Lys\textsuperscript{24}-Ile\textsuperscript{25} is required to generate active KLK14, a process that may be performed by KLK5 (Brattsand et al., 2005). Despite the enzyme’s predicted trypsin-like substrate specificity with a preference for basic P1 residues, it has been shown that KLK14 can possess dual, trypsin-like and chymotrypsin-like, substrate specificity (Brattsand et al., 2005; Felber et al., 2005). The proteinase can regulate its activity by autolytic cleavage leading to enzymatic inactivation (Borgoño et al., 2007c). Further regulation is achieved by inhibitory serpins, such as α1-antitrypsin, α2-antiplasmin, antithrombin III, and α1-antichymotrypsin (Luo and Jiang, 2006; Borgoño et al., 2007c), as well as by the Kazal-type inhibitor LEKTI (Borgoño et al., 2007b). In
addition, citrate and zinc ions can exert stimulatory or inhibitory effects, respectively, on the activity of KLK14 (Borgoño et al., 2007c).

Phage-displayed penta-peptide motifs preferred by KLK14 were identified within several intact proteins, including the extracellular matrix (ECM) molecules laminin, collagen type IV, and matrilin-4 (Felber et al., 2005). The *in vitro* target repertoire of KLK14 has been recently expanded to include collagens type I–IV, fibronectin, laminin, kininogen, fibrinogen, plasminogen, vitronectin, and insulin-like growth factor-binding proteins 2 and 3 (Borgoño et al., 2007c). These findings have led to the hypothesis that KLK14 may be implicated in several phases of tumour progression, including growth, invasion, and angiogenesis, as well as in arthritis via deterioration of cartilage.

Several studies have demonstrated that the *KLK14* gene is under steroid-hormone regulation (Borgoño et al., 2003; Yousef et al., 2003a) and is most highly expressed in the glandular epithelia of the breast (Borgoño et al., 2003, Fritzsche et al., 2006) and prostate (Hooper et al., 2001; Borgoño et al., 2003). In addition, it is found in the epidermis (*i.e.* stratum granulosum) and appendages of the skin (Borgoño et al., 2003, Komatsu et al., 2003, Stefansson et al., 2006). Consequent to its secretion, KLK14 remains in seminal plasma (Borgoño et al., 2003) and sweat (Komatsu et al., 2006b) and is present in the stratum corneum of the skin (Komatsu et al., 2005; Komatsu et al., 2006b) in its catalytically active form (Stefansson et al., 2006). Furthermore, aberrant KLK14 expression has been detected in patients with breast (Yousef et al., 2001; Borgoño et al., 2003; Fritzsche et al., 2006; Yousef et al., 2002a), ovarian (Yousef et al., 2001; Borgoño et al., 2003; Yousef et al., 2003a), prostate (Yousef et al., 2001; Yousef et al., 2003f), and testicular (Yousef et al., 2001) cancer, as well as patients with peeling skin syndrome (Komatsu et al., 2006a). Correlative clinical data have also linked elevated *KLK14* with aggressive forms of breast (Fritzsche et al., 2006, Yousef et al.,
2002a) and prostate cancer (Yousef et al., 2003f) and with the prognosis of breast (Yousef et al., 2002a) and ovarian (Yousef et al., 2003a) cancer patients. Possibly as part of a KLK cascade (e.g. KLKs 5 and 7), KLK14 has also been linked to epidermal desquamation due to degradation of the intercellular (corneo)desmosomal adhesion molecules that link adjacent corneocytes (Caubet et al., 2004; Brattsand et al., 2005; Komatsu et al., 2005; Stefansson et al., 2006; Komatsu et al., 2006a; Borgoño et al., 2007b), as well as to a brain cascade along with several other KLKs (Yoon et al., 2007). Finally, a recent study from our group has linked KLK14 to the seminal plasma cascade, activation of which is initiated upon ejaculation (Emami et al., 2008; Emami and Diamandis, 2008).

**Table 7: Basic properties of kallikrein-related peptidases 5, 6, and 14.** The abbreviations used indicate: α2-AP = α2-antiplasmin, AT III = antithrombin III, α1-AT = α1-antitrypsin, α1-ACT = α1-antichymotrypsin.

<table>
<thead>
<tr>
<th>KLK</th>
<th>Preference</th>
<th>Activation</th>
<th>De-activation</th>
<th>Inhibitors</th>
<th>Significant expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK5</td>
<td>Arg &gt; Lys</td>
<td>Auto?</td>
<td>Unknown</td>
<td>α2-AP, AT III</td>
<td>Skin, seminal plasma, prostate cancer</td>
</tr>
<tr>
<td>KLK6</td>
<td>Arg &gt; Lys</td>
<td>Auto?</td>
<td>Auto?</td>
<td>α2-AP, AT III, α1-ACT</td>
<td>Brain, ovarian cancer</td>
</tr>
<tr>
<td>KLK14</td>
<td>Arg &gt; Lys</td>
<td>KLK5?</td>
<td>Auto</td>
<td>α1-AT, α2-AP, AT III, α1-ACT</td>
<td>Skin, seminal plasma, breast cancer</td>
</tr>
</tbody>
</table>

Table 7: Basic properties of kallikrein-related peptidases 5, 6, and 14. The abbreviations used indicate: α2-AP = α2-antiplasmin, AT III = antithrombin III, α1-AT = α1-antitrypsin, α1-ACT = α1-antichymotrypsin.
Figure 6: General structural features of KLKs 5, 6, and 14. Upon removal of its signal peptide the KLK proform is released to the extracellular micro-environment (proteolysis occurs after the single-letter code-indicated amino acids). Proteolytic cleavage of the proform (proteolysis after R or K, as indicated) results in the formation of a mature protein with 223-227 amino acids (aa). The single letters represent the amino acids preceding the cleavage sites.

2.2 Material and Methods

2.2.1 Reagents

Diflunisal phosphate (DFP) was synthesized in our laboratory. The stock solution of DFP was 0.01 M in 0.1 M NaOH. Thermolysin was obtained from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada; catalogue number T7902; 44 Units/mg solid or 77 Units/mg protein; 1 Unit hydrolyzes casein to produce colour equivalent to 1 μmol / min at pH 7.5 and 37ºC). Porcine trypsin was also purchased from Sigma (Sigma, catalogue number T-7418; 14 900 Units/mg). The probes that were used to detect serine proteinase activity (ABP), were provided by Dr. Amos Baruch (biotin-Pro-Lys-diphenylphosphonate or Bio-PK; KAI Pharmaceuticals, South San Francisco, CA, USA; Pan et al., 2006) and by Dr. Brendan Gilmore
(biotin-Suc-Lys-diphenylphosphonate; School of Pharmacy, Queens University Belfast, Medical Biology Centre, Belfast, UK; Hamilton et al., 1993; Hawthorne et al., 2004; Gilmore et al., 2006; unpublished data). The probes were designed to specifically target the active site of serine proteinases with trypsin-like activity. The Bio-PK probe was mainly used for the work described hereafter, unless otherwise stated. The enzymatic activity of KLK6 was also measured as previously described, using the fluorogenic synthetic substrate t-butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin (VPR-AMC; Bachem Bioscience Inc., King of Prussia, PA, USA) (Magklara et al., 2003). The enzymatic activity reaction was performed in 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween-20, pH 7.6, at 37ºC. Similarly, the activity of KLK5 against VPR-AMC was estimated using 100 mM Na₂HPO₄, 0.01% Tween-20, pH 8.0 (Michael et al., 2005). The enzymatic activity of KLK14 was estimated utilizing a buffer identical to this described for KLK5, while t-butoxycarbonyl-Gln-Ala-Arg-7-Amino-4-methylcoumarin (QAR-AMC; Bachem Bioscience) was used as substrate (Borgoño et al., 2007c).

Fluorescence was monitored for 20 min on a Wallac Victor fluorometer (PerkinElmer Life Sciences, Wellesley, MA, USA) set at 355 nm for excitation and 460 nm for emission. The fluorescence values of enzyme-free reactions were used as negative controls and background fluorescence was subtracted from each value. All experiments were performed in triplicate. A standard curve with known concentrations of AMC was used to calculate the rate of product formation. Kinetic analysis was done by nonlinear regression analysis using the Enzyme Kinetics Module 1.1 (Sigma Plot, SSPS, Chicago, IL, USA).
2.2.2 Immunofluorometric quantification of KLKs 5, 6, and 14

In the case of the enzyme-linked immunosorbent assay (ELISA), which utilizes 2 monoclonal antibodies, white polystyrene 96-well microtiter plates were coated with anti-KLK monoclonal antibody (Figure 7); coating antibody solution (100 µL of 50 mM Tris buffer, 0.05% sodium azide, pH 7.8, containing 500 ng/well antibody) was added to each well and incubated overnight at room temperature. Calibrators were prepared by diluting recombinant KLK protein produced as described in the later sections in bovine serum albumin (BSA) buffer (6% BSA, 50 mM Tris, 0.05% sodium azide, pH 7.8). Where appropriate, sample dilutions were also performed in 6% BSA buffer. The plates were washed two times with the washing buffer (5 mM Tris buffer, 150 mM NaCl, 0.05% Tween-20, pH 7.8). Protein calibrators or samples were added to each well (100 µl/well) after being diluted 2-fold in assay buffer [50 mM Tris, 6% BSA, 0.01% goat IgG (Sigma), 0.005% mouse IgG (Fortron Bio Science Inc, Morrisville, NC), 0.1% bovine IgG (Sigma), 0.5 M KCl, 0.05% sodium azide, pH 7.8] and were incubated for 2 h with shaking at room temperature. The plates were washed six times with the washing buffer and 100 µL of biotinylated detection antibody solution (25-50 ng/well of anti-KLK monoclonal antibody in assay buffer) was added to each well and incubated for 1 h at room temperature with shaking. The plates were then washed six times with the washing buffer. Subsequently, 5 ng/well of streptavidin-alkaline phosphatase (SA-ALP) solution (Jackson ImmunoResearch, West Grove, PA, USA) in BSA buffer was added to each well and incubated for 15 min at room temperature. The plate was washed six times with washing buffer and the substrate (100 ml of 0.1 M Tris buffer, pH 9.1, containing 1 mM DFP, 0.1 M NaCl, and 1 mM MgCl₂) was added to each well and incubated for 10 min at room temperature. Finally, developing solution (100 ml of 1 M Tris, 0.4 M NaOH, 2 mM TbCl₃, 3 mM EDTA) was added to each well and incubated for 1 min at room temperature. The Tb³⁺ fluorescence was measured on a time-resolved
fluorometer, the Cyberfluor 615 Immunoanalyser (MDS Nordion, Kanata, ON, Canada). Calibration and data reduction were performed automatically. The assay principle has been described in detail elsewhere (Christopoulos and Diamandis, 1992; Ferguson et al., 1996).

In the sandwich-type monoclonal-polyclonal ELISA (Figure 7) the microtiter plates were coated with KLK monoclonal antibody and processed as above. Detection involved addition for 1 h of a rabbit anti-KLK14 polyclonal antiserum diluted in assay buffer. The alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), diluted 2000-fold in washing buffer was additionally added to each well, incubated for 45 min, washed, and detected as above. Unless stated otherwise all reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

![Diagram of ELISA principle](image)

**Figure 7: Main principle of the mono-mono and mono-poly configuration of ELISA.** The biotinylated antibody is represented with B and ALP stands for alkaline phosphatase conjugated with streptavidin (SA). Adding diflunisal phosphate (DFP), which is a substrate of ALP, increases the release of fluorescence measured by the Cyberfluor Immunoanalyser.
The detection limit of these immunoassays and the cross-reactivity with other kallikrein-related peptidases has been studied, suggesting that these assays are both highly sensitive and specific (Table 8).

Table 8: Immunofluorometric quantification of kallikrein-related peptidases 5, 6, and 14.

<table>
<thead>
<tr>
<th>KLK</th>
<th>Assay configuration</th>
<th>Detection limit (µg/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK5</td>
<td>Mono-mono</td>
<td>0.05</td>
<td>Diamandis et al., 2003; Shaw and Diamandis, 2007</td>
</tr>
<tr>
<td>KLK6</td>
<td>Mono-mono</td>
<td>0.05</td>
<td>Diamandis et al., 2000d; Luo et al., 2006</td>
</tr>
<tr>
<td>KLK14</td>
<td>Mono-poly</td>
<td>0.05</td>
<td>Borgoño et al., 2007c; Shaw and Diamandis, 2007</td>
</tr>
</tbody>
</table>

2.2.3 Production, purification, and activation of kallikrein-related peptidase 5 and 14

KLK5 zymogen, expressed in *Pichia pastoris* (strain KM71), was purified, as previously described (Michael et al., 2005). Yeast culture supernatant was clarified by centrifugation and concentrated 10-fold by using a Centricon ultrafiltration device (Millipore, Waltham, MA, USA) with a 10-kDa molecular weight cutoff nitrocellulose membrane (Millipore). Sample was then diluted 2-fold with running buffer (50 mM sodium acetate, pH 5.3). Purification of KLK5 was achieved with cation-exchange fast performance liquid chromatography (FPLC); the concentrated yeast supernatant was loaded onto a 5-ml CM FF-Sepharose cation exchange column (Amersham Biosciences). The column was eluted with 50 mM sodium acetate and 1 M NaCl, pH 5.3, with a multistep gradient of 0-1 M NaCl at a rate of 2.5 ml/min.

The active form of KLK14 was expressed in *Pichia pastoris* (strain X-33), as described previously (Felber et al., 2005). Concentrated yeast supernatant was clarified by centrifugation and concentrated 10-fold by using a Centricon ultrafiltration device (Millipore, Waltham, MA, USA) with a 10 kDa molecular weight cutoff nitrocellulose membrane (Millipore), diluted 2-fold with running buffer (10 mM MES, pH 5.3). The supernatant was loaded onto a 5-ml
HiTrap SP HP-Sepharose cation exchange column (Amersham Biosciences). The column was eluted with 10 mM MES buffer and 1 M KCl, pH 5.3, with a multistep gradient of 0-1 M KCl at a rate of 3 ml/min.

In both cases FPLC fractions were analyzed by specific enzyme-linked immunosorbent assay, VPR-AMC (KLK5) or QAR-AMC (KLK14) activity analysis and SDS-PAGE under reducing conditions (NuPage 4–12% Bis-Tris gels; Invitrogen, Burlington, ON, Canada), followed by coomassie staining (SimplyBlue Safestain; Sigma). Fractions containing the active enzyme were pooled and concentrated in 50 mM sodium acetate (KLK5) or 10 mM MES (KLK14), pH 5.3. The concentration of the purified catalytically active recombinant protein was determined by enzyme-linked immunosorbent assay and total protein assay. The purity of the protein (>90%) was verified by SDS-PAGE gel electrophoresis and coomassie staining.

2.2.4 Production of active kallikrein-related peptidase 6 in baculovirus

The recombinant active KLK6, which was used for the experiments of Chapter III, was kindly provided by Dr. Michael Blaber (Department of Biomedical Sciences, Florida State University, Tallahassee, FL, USA). The enzyme was produced in a baculovirus/insect cell line system and activated by enterokinase, as previously described (Blaber et al., 2002; Yoon et al., 2007; Li et al., 2008). In brief the cDNA encoding the sequence for the mature form of KLK6 was inserted into the pBAC3 transfer vector (Novagen, Madison, WI, USA) immediately 3’ to a synthetic pre-pro-sequence containing a secretion signal, a His-tag, and the enterokinase recognition sequence of (Asp)₄Lys. This resulted in a 44 amino acid synthetic pro-sequence, which ended in the EK recognition sequence and was followed by the amino-terminal sequence of the mature form of KLK6. Expression of KLK6 in the pBAC3 transfer vector utilized the BacVector transfection system (Novagen). The Sf9 insect cell line, in conjunction with Sf-900
II serum-free media (Life Technologies, Rockville, MD, USA), was used for preparation of high-titer (i.e., >109 pfu/mL) viral stock. The TN5 (High5; Invitrogen Corp., Carlsbad, CA, USA) insect cell line was used for production of expressed protein by the viral stock. The recombinant protein was purified in a single step utilizing the His-tag fusion and nickel affinity resin (Ni-NTA). Fractions containing KLK6 were pooled and extensively dialyzed versus 40 mM sodium acetate, pH 4.5, using 6-8 kDa molecular mass cutoff dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA, USA). The purity and integrity of the protein was verified by amino-terminal sequencing, mass spectrometry, and size exclusion high-performance liquid chromatography (HPLC).

The recombinant protein was subjected to enterokinase digestion (Roche Diagnostics Corp., Indianapolis, IN, USA). Separation of the pro-sequence and enterokinase from mature KLK6 was performed using Sephadex G-50 gel filtration (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA) in 40 mM sodium acetate and 100 mM NaCl, 0.01% Tween-20, pH 4.5. The protein was lyophilized to dryness and resuspended into 10 mM sodium acetate, 0.01% Tween-20, pH 5.2 to prevent protein degradation. The enzyme activity was evaluated by VPR-AMC activity analysis. The integrity of the preparation was determined by SDS-PAGE gel electrophoresis (under reducing conditions) and coomassie staining. Purified KLK6 was a single chain form with an approximate molecular mass of 26 kDa (according to MALDI-MS and SDS-PAGE gel electrophoresis).

2.2.5 Production, purification, and activation of KLK6 in HEK-293 cells

Human embryonic kidney (HEK-293) cells, transfected with the zymogen form of KLK6, were grown in serum-free medium, as previously described (Little et al., 1997; Magklara et al., 2003). Pure KLK6 zymogen (inactive proform) was purified as described by
Magklara et al. (2003) with minor protocol modifications. In brief, the tissue culture supernatant was collected and concentrated 10-fold by using a Centricon ultrafiltration device (Millipore, Waltham, MA, USA) and a 10-kDa molecular weight cutoff nitrocellulose membrane (Millipore). Concentrated supernatant was then diluted 2-fold in running buffer (50 mM sodium acetate, pH 5.3) and pH was adjusted to 5.3, before analyzing the sample by ion-exchange liquid chromatography (ÄKTA™FPLC; GE Healthcare Bio-Sciences Corp.) using either Protocol A, for production of KLK6 zymogen and subsequent conversion to its active form, or Protocol B, for direct production of active KLK6 from cell culture supernatants.

**Protocol A. Production of KLK6 zymogen**

To purify the zymogen of KLK6 we loaded the concentrated supernatant onto an ion exchange column (5-ml HiTrap CM FF column, GE Healthcare Bio-Sciences Corp.), as previously described (Magklara et al., 2003). Fractions obtained from FPLC were analyzed by enzyme-linked immunosorbent assay and those containing KLK6 were pooled, supplemented with 1% trifluoroacetic acid, loaded onto a 1-ml C4 reverse-phase column (VYDAC® 214TP; Grace, Deerfield, IL, USA), and separated by reverse-phase high performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies Inc. Santa Clara, CA, USA), using 0.1% trifluoroacetic acid in deionised H₂O. Elution was performed with acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA), using a linear gradient of 0-100% ACN.

Activation of the KLK6 zymogen was achieved by incubation for up to 2 h with thermolysin at a 1:115 molar ratio (thermolysin/KLK6) in 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, pH 7.5, at room temperature. The reaction was terminated with the addition of 50 µM EDTA followed by rapid freezing with liquid nitrogen. The conversion from proform to active enzyme was monitored by incubation with 5 nM of the biotin-linker-Pro-Lys-
diphenylphosphonate probe (Bio-PK) for 2 h, at room temperature, and analysis with SDS-PAGE gel electrophoresis under reducing conditions (NuPage 4–12% Bis–Tris gels; Invitrogen, Burlington, ON, Canada). Biotinylated proteins were transferred to a Hybond-C Extra (Amersham Biosciences, Pittsburgh, PA, USA) nitrocellulose membrane and blocked with 5% casein and 0.1% Tween-20 in Tris buffered saline (TBST) for 2 h, at room temperature. Subsequently, they were washed briefly with TBST, treated with streptavidin-alkaline phosphatase (SA-ALP; 50 ng/ml SA-ALP, diluted in 1% casein-TBST) for 1 h, washed, and visualized with Immulite Chemiluminescent substrate treatment for 10 min (Diagnostic Products Corp, Los Angeles, CA). This western blotting detection of activity-based probe-proteinase complexes has been previously described (Hansen et al., 2005).

**Protocol B. Direct isolation of active KLK6**

To purify active KLK6 directly from concentrated cell culture supernatant we used the ion-exchange liquid chromatography protocol as described above, slightly modified to include a new two-step chromatographic protein isolation procedure with a salt-step sodium chloride gradient (% v/v of 1 M NaCl in 50 mM sodium acetate buffer, pH 5.3 as detailed below). Concentrated supernatant was diluted 2-fold in running buffer as above and loaded onto the FPLC column (5-ml HiTrap SP HP column, GE Healthcare Bio-Sciences Corp.) at a flow rate of 0.8 ml/min. Four-milliliter fractions (3 ml/min) were eluted by using a three-step gradient of 10% (15 column volumes), 20% (15 column volumes), 30% (10 column volumes) of elution buffer (1 M NaCl in 50 mM sodium acetate, pH 5.3). Fractions were analyzed with ELISA and SDS-PAGE gel electrophoresis under reducing conditions. Protein in the SDS-PAGE gels was monitored (data not shown) by western blot (KLK6 antibody), coomassie stain (SimplyBlue Safestain; Sigma), and by Bio-PK labeling as described in Protocol A. The enzymatic activity of
fractions was also assessed using the fluorogenic synthetic substrate VPR-AMC (Bachem Bioscience), as previously described (Magklara et al., 2003).

Fractions containing the zymogen (western blot and enzymatic activity analysis) of KLK6 were pooled and incubated with 5.8 Units of thermolysin (75 µg) at a 1:115 thermolysin to KLK6 molar ratio. The reaction was allowed to proceed for 2 h at room temperature and at pH 7.5. Following incubation, the sample was diluted in running buffer, the pH was adjusted to 5.3 and the sample was loaded onto the 5-ml HiTrap SP HP FPLC column. The identical salt-step gradient was run and FPLC fractions were collected and analyzed as described above. Fractions were also incubated with 5 nM of the Bio-PK probe, as in Protocol A, to monitor serine proteinase activity (Hansen et al. 2005). The fractions containing active KLK6 were combined and concentrated 10-fold in 50 mM sodium acetate, pH 5.3, using 10-kDa molecular weight cutoff Amicon concentration tubes (Millipore). Protein purity was accessed with SDS-PAGE gel electrophoresis under reducing conditions and detailed enzyme kinetic analysis was performed with VPR-AMC (Magklara et al., 2003).

2.2.6 Storage optimization of the newly produced active KLK6

Active KLK6 produced under Protocol B was incubated at room temperature, 4°C, -20°C, and -80°C to evaluate the stability of the protein and to ensure retention of optimal enzyme activity for the purpose of our experiments. Incubation was preformed in 50 mM sodium acetate, pH 5.3 at given temperatures for up to 12 months. Aliquots were removed at specific time points and detailed enzymatic activity was performed, as described above.

We also incubated KLK6 with the N-glycosidase PNGase F (New England Biolabs Ltd., Pickering, ON, Canada) to investigate the enzyme activity upon removal of its glycan moiety. De-glycosylation was performed in a 40 µl reaction volume containing 4 µg of KLK6 and 4
Units of N-glycosidase F. Having observed that denaturing conditions greatly reduce the enzyme activity of KLK6, we selected to perform incubation at 50 mM Tris-HCl, pH 7.5, at 37°C and for up to 24 hours, without prior denaturation of the recombinant proteins, as suggested previously (Bayés et al., 2004). The enzyme activity of KLK6 was assessed by VPR-AMC analysis and the reaction products were resolved by SDS-PAGE. We compared our findings with the level of degradation that was observed during the thermolysin incubation in Protocol A, as well as with the previously reported stability of KLK6 at similar basic pH levels (Magklara et al., 2003; Bayés et al., 2004; Blaber et al., 2007).

2.3 Results

2.3.1 Recombinant active KLK5 and KLK14

We took advantage of the available assays to monitor the enzymatic activity of KLKs to re-evaluate the already established protocols for the production and purification of KLKs 5 and 14 from yeast supernatant. This procedure for KLK5 production directly yielded the four expected glycosylated forms of KLK5, which were all active, due to auto-activation or activation by an unknown yeast proteinase (Figure 8, white arrow). Purification of KLK14 resulted in the isolation of catalytically active enzyme (>80% active) amongst other molecular weight species (Figure 8). KLK14 existed mainly as 2 forms with different molecular weights (labeled as 1 and 2 in Figure 8A), which were easily separated via FPLC. Interestingly, only form 2 (bolded arrow) was highly active against the QAR-AMC substrate (approx. 20-fold higher activity as determined by detailed enzyme kinetics; data not shown) and the biotin-Suc-Lys-diphenylphosphonate activity probe (Figure 8B).
2.3.2 Production, purification, and activation of KLK6 zymogen in HEK-293 cells

KLK6 zymogen, with minimal detectable enzymatic activity against VPR-AMC, was isolated by reverse-phase chromatography, as previously described (Magklara et al., 2003) and the recombinant protein was activated with thermolysin. Control samples lacking thermolysin were incubated under the same conditions. After a 2-h incubation, more than 99% of the KLK6 zymogen (Figure 9, upper panel, solid arrowhead) was converted to a form with a lower molecular weight and greater mobility on SDS-PAGE gels, as detected by the coomassie protein stain (Figure 9, upper panel, open arrowhead). Within the same time frame, we found no evidence for further cleavage of the enzyme (due to non-specific proteolytic activity of thermolysin or to KLK6 auto-degradation) according to the electrophoretic analysis of the
protein with either coomassie (Figure 9, upper panel) or silver stain protein detection (Figure 9, lower panel). Western blot analysis of the same samples revealed faint lower molecular weight bands of about 26 kDa, possibly representing KLK6 degradation fragments (Figure 9, second panel). These minor fragments that may have been generated in the course of thermolysin activation were not labeled by the activity-based probe (Figure 9, compare second and third panels) and, therefore, represented immunoreactive enzymatically inactive fragments. Such inactive degradation products have been previously reported in the literature (Bernett et al., 2002; Magklara et al., 2003; Bayés et al., 2004). Activity-based labeling using the biotin-linker-Pro-Lys-diphenylphosphonate probe (Bio-PK) revealed a very rapid processing of the zymogen, with increasing levels of catalytically active KLK6 appearing over the two-hour time frame of incubation (Figure 9, left part of third panel). A very low, but detectable level of residual enzyme activity was also found in samples that were not treated with thermolysin (Figure 9, right part of third panel).
Figure 9. Monitoring activation of recombinant KLK6 by thermolysin. Activation of the zymogen was achieved as outlined in the text over a two-hour time period, with termination of the reaction by the addition of 50 mM EDTA. The reaction proceeded without the appearance of KLK6 degradation fragments (silver stain detection: lower panel). Conversion of the zymogen to active enzyme was monitored by SDS-PAGE analysis followed by detection of KLK6 by: (1) Upper panel, coomassie stain, (2) Second panel, western blot (3) Third panel, activity-based probe labeling followed by streptavidin-alkaline phosphatase visualization, and (4) Lower panel, silver stain (lanes 1-7). The-23 kDa molecular weight band in the third gel shows the activity-based probe labeling of 100 ng porcine trypsin (Trp). The table below the gel images provides the details for each analytic lane designated by the numbers at the top of the gels. Active KLK6 (open arrowhead, upper panel) migrated more rapidly in the gel than the higher molecular weight zymogen (solid arrowhead, upper panel). Thermolysin treatment rapidly activated the enzyme within the time-frame of sample preparation (lane 1, third panel), as shown by the reaction with biotinylated activity-based probe visualized with streptavidin-alkaline phosphatase. The ABP reacts ONLY with active KLK6.
2.3.3 Production and purification of active KLK6 protein

We exploited the ability of thermolysin to activate KLK6 rapidly and efficiently to develop a single method of enzyme purification and activation, directly from the KLK6-expressing HEK-293 cell supernatants. The method included two rounds of ion-exchange liquid chromatography purification (FPLC), separated by the activation step, as described in detail in the Materials and Methods section. A representative second-round chromatogram of FPLC with and without the step of KLK6 activation by thermolysin is shown in Figure 10A. FPLC fractions were reacted with the Bio-PK probe, resolved by SDS-PAGE under reducing conditions and detected by streptavidin-based western blotting to detect serine proteinases that had reacted with the biotinylated activity-based probe (Figure 10B, lower panel). This analysis proved that the FPLC-thermolysin activation-FPLC method can yield high levels of pure and active KLK6, which was visualized separately as the major component detected by coomassie protein staining (Figure 10B, upper panel).
Figure 10: Production of active KLK6; FPLC chromatography of zymogen and thermolysin-activated enzyme (A) and SDS-PAGE analysis of protein and ABP-labelled enzyme (B). Zymogen preparation was harvested from cell supernatants by FPLC as outlined in Protocol B in Materials and Methods. Fractions containing KLK6 immunoreactivity (western blot: not shown) with no detectable enzymatic activity (VPR-AMC analysis: not shown) were pooled, treated or not with thermolysin, and subjected to a second round of FPLC (A) using a salt-step gradient, as described in Materials and Methods (lines above/through the UV tracings in chromatograms of Panel A). The top chromatogram shows a representative FPLC chromatogram (UV Absorbance at 280 nm) of a KLK6 zymogen preparation, without prior activation with thermolysin. Fractions were monitored for KLK6 immunoreactivity and five groups of pooled samples were collected (Groups 1 to 5). The markings over the X-axis represent the FPLC fraction numbers. The lower chromatogram of thermolysin-activated KLK6 is the result of a salt-step gradient that was the same as for the upper chromatogram. The fractions containing active enzyme, eluting between about 65 and 75 minutes (monitored by western blot detection and activity-based labelling: not shown for individual fractions) were pooled (Group 4). In total, five pooled fraction samples were obtained from each panel (Groups 1 to 5) and then analyzed further by SDS-PAGE (B) or incubated with the Bio-PK. Proteins in the gels were detected either by coomassie stain (upper gels) or with SA-ALP used to visualize the biotin-tagged enzymes (lower gels). The positions of the molecular weight markers (kDa) are shown on the right. The active enzyme bands are denoted by the open arrowheads (upper and lower gels); inactive zymogen is designated by the solid arrowhead (upper gels). Lower molecular weight bands are likely the result of protein degradation as discussed in the text. The 23-kDa band in the bottom gel shows the SDS-PAGE analysis of 100 ng ABP-reactive porcine trypsin (Trp).
2.3.4 Stability of active KLK6 protein

The active KLK6 preparation was stable in terms of enzymatic activity when stored in 50 mM sodium acetate buffer, pH 5.3. The storage optimization analysis showed that > 90% of KLK6 activity was retained when protein was incubated for close to 60 h at room temperature; however, storage at either 4ºC or -20ºC greatly increased the half life of active KLK6, while the activity of KLK6 in -80ºC was retained for more than a year. Results for up to 5 months are presented in Table 9 and Figure 11.

Table 9: Storage optimization of active KLK6 in 50 mM sodium acetate buffer, pH 5.3.

<table>
<thead>
<tr>
<th>Time*</th>
<th>Activity in nmol VPR-AMC substrate / min / mg KLK6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT</td>
</tr>
<tr>
<td>&lt; 0 min</td>
<td>1709.424</td>
</tr>
<tr>
<td>0 min</td>
<td>1392.831</td>
</tr>
<tr>
<td>3 hours</td>
<td>2389.857</td>
</tr>
<tr>
<td>20 hours</td>
<td>2008.833</td>
</tr>
<tr>
<td>64 hours</td>
<td>1567.576</td>
</tr>
<tr>
<td>2 days</td>
<td>1239.569</td>
</tr>
<tr>
<td>9 days</td>
<td>985.3626</td>
</tr>
<tr>
<td>25 days</td>
<td>633.1201</td>
</tr>
<tr>
<td>1 week</td>
<td>1847.275</td>
</tr>
<tr>
<td>2 weeks</td>
<td>1515.827</td>
</tr>
<tr>
<td>1 months</td>
<td>1650.006</td>
</tr>
<tr>
<td>2 months</td>
<td>1441.358</td>
</tr>
<tr>
<td>3 months</td>
<td></td>
</tr>
<tr>
<td>4 months</td>
<td>1083.826</td>
</tr>
<tr>
<td>5 months</td>
<td>1140.155</td>
</tr>
</tbody>
</table>

* Protein was stored in -80ºC for one week between time “<0” and time “0”, when the different aliquots were created and used to investigate the levels of activity at indicated time points. The observed decrease in protein activity between the two time points indicated above may be the result of the freezing-thawing effect on enzyme activity.
We de-glycosylated KLK6 with the N-glycosidase PNGase F and concluded that after 8 hours of incubation at 37°C, both the glycosylated as well as the non-glycosylated enzyme had lost around 30% of its activity. The rate of decrease in enzyme activity was similar for both forms of KLK6. This experiment confirmed previous findings that glycosylation of KLK6 does not directly effect its activity (Bayés et al., 2004). We also confirmed the finding of Magklara et al. (2003) that KLK6 enzymatic activity is relatively stable for about 2 hours at pH 7.5, 37°C.
The enzyme activity of KLK6 against VPR-AMC at different time points of incubation with PNGase F is shown in Figure 12. In addition, the end products after a 24-hour incubation were resolved by SDS-PAGE and blotted with an in-house anti-KLK6 polyclonal antibody, as shown in Figure 12.

**Figure 12: Activity of the de-glycosylated form of KLK6.** Active KLK6 is denoted by the white arrows (molecular weights of about 28 and 30 kDa, for the non-glycosylated and glycosylated KLK6, respectively), whereas the bulky lower molecular weight bands, at 24 h of incubation at 37°C, are the products of protein degradation, as previously reported by our group and others (Bernett *et al.*, 2002; Magklara *et al.*, 2003; Bayés *et al.*, 2004).
2.4 Conclusions

Our group has described the production and purification of active KLK5 and KLK14 in previous publications (Michael et al., 2005; Borgoño et al., 2007c). Nevertheless, the new analytical tools that became available, namely the serine proteinase activity-based probes (ABP), as well as the fluorogenic peptides, allowed us to optimize the production of fully active KLKs. Our study showed for the first time that yeast supernatant contains 2 forms of KLK14 (labeled as 1 and 2 in Figure 8A), with significantly different enzymatic activity.

Moreover, thus far, our lab had not been able to produce active KLK6, despite the high protein yield offered by our systems and the proposed mechanism of auto-activation of this enzyme (Magklara et al., 2003). However, given the importance of kallikrein-related peptidase 6 to diseases, such as ovarian cancer and Alzheimer’s disease, the need for production of high levels of pure and active KLK6 has been rapidly increasing. It should be mentioned that active KLK6 had been previously generated from three different sources: (a) from zymogen purified from KLK6-transfected yeast supernatant and treated with lysil endoprotease (Bayés et al., 2004), (b) upon activation of KLK6, containing a synthetic pro-sequence recognized and cleaved by enterokinase, produced in a baculovirus system (Blaber et al., 2002; Yoon et al., 2007), and (c) from enterokinase activation of KLK6, produced in E. coli (Debela et al., 2006). The enzyme produced using the second system mentioned above was utilized for the pharmacological study of KLK6 described in Chapter III. However, to expand our analysis, we aimed to isolate active KLK6 from cell culture supernatants of HEK-293 cells transfected with the zymogen of KLK6. This production system would additionally ensure the proper folding and glycosylation of the enzyme within the mammalian host cell, an asset which is very important when studying the in vivo function of an enzyme.
Previous protocols using the HEK-293 mammalian system depended on the auto-activation properties of KLK6 for generation of the active form (Little et al., 1997, Magklara et al., 2003). However, a recent study indicated that the rate of KLK6 auto-activation is slower than its rate of auto-degradation (Blaber et al., 2007). We therefore elected to use thermolysin to accelerate the activation of KLK6 over its degradation.

As it was the case for KLKs 5 and 14, the focus of this exercise was to use the serine proteinase activity-based probes (ABP) and the fluorogenic peptides to monitor the production of active KLK6. This approach (e.g. ABP labelling coupled to western blot detection and VPR-AMC analysis) facilitated the development of an efficient method for the purification of fully active KLK6 from a cell expression system that secretes significant amounts of zymogen amongst a variety of other proteins in the serum-free supernatant. The two-step procedure, involving a thermolysin-catalyzed activation of KLK6 as monitored by the ABP reaction, takes advantage of the FPLC chromatographic shift in elution of the active enzyme relative to the KLK6 zymogen. This shift to higher elution volume of the activated enzyme (e.g. fractions eluting between 65 and 75 min, Figure 10A, lower chromatogram), relative to the zymogen-containing fractions recovered in the first step, frees the thermolysin-generated product from contaminating proteins that elute at the position of the zymogen in the first step (e.g. fractions eluting between 40 and 65 min, Figure 10A, upper chromatogram). Fortuitously, removal of thermolysin from the incubation mixture was possible during the FPLC fractionation, due to the different chemical properties of thermolysin. This straightforward two-step FPLC procedure provided the key active-enzyme preparation that allowed us to expand our studies.

In summary, this part of the work focused on the production of active KLKs 5, 6, and 14 in quantities sufficient to allow us to proceed with our hypothesis of proteinase-activated receptor activation by kallikrein-related peptidases.
Chapter III.

Interaction of KLKs 5, 6, and 14 with PARs

ORIGINAL PUBLICATIONS:


INTERACTION OF KLKs 5, 6, AND 14 WITH PARs

3.1 Introduction

As mentioned in the general Introduction section, proteinase-activated receptors (PARs 1-4) compose a unique family of four G-protein-coupled cell surface receptors for certain proteinases (Rasmussen et al., 1991; Vu et al., 1991; Nystedt et al., 1994; Macfarlane et al., 2001; O’Brien et al., 2001; Hollenberg and Compton, 2002; Ossovskaya and Bunnett, 2004; Coughlin, 2005; Steinhoff et al., 2005). Proteolytic cleavage within the extracellular N-terminus reveals a tethered ligand that binds to the extracellular receptor domains to initiate cell signalling (Macfarlane et al., 2001; Hollenberg and Compton, 2002; Ossovskaya and Bunnett, 2004; Seatter et al., 2004). Proteinases that activate PARs include coagulation factors, enzymes from inflammatory cells, and proteinases from epithelial cells and neurons. These enzymes, which are generated and released during injury and inflammation, can cleave and activate PARs on many cell types from a variety of species (humans, rats, and mice) to regulate critically important processes, such as haemostasis, inflammation, pain, and tissue repair. Other proteinases that cleave PARs downstream of the N-terminal tethered ligand sequence disable the receptors for further proteolytic activation, thus abrogating PAR signalling. 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, neurodegeneration, and cancer (Vergnolle et al., 2001b; Hollenberg and Compton, 2002; Noorbakhsh et al., 2003; Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005).

In some systems, the proteinases that activate PARs have been established. For example, in the coagulation system, PAR₁, PAR₃, and PAR₄ are recognized physiological targets for
thrombin (Coughlin, 2005), which does not efficiently activate PAR₂. However, PAR₂ can be activated by the complex of tissue factors VIIa and Xa (Ruf et al., 2003). Furthermore, plasmin can have a complex action on PARs as it can both activate and dis-arm PAR₁ (Kimura et al., 1996; Kuliopulos et al., 1999), while it can activate PAR₄ (Quinton et al. 2004). Finally, activated protein C, via the endothelial adsorption site and a targeted interaction involving an exosite domain on activated protein C can activate PAR₁ (Riewald et al., 2002; Yang et al., 2007).

In the gastrointestinal tract, trypsin (presumably, trypsin-1 or -2 in humans) can activate PAR₂ on enterocytes (Kong et al., 1997). Elevated levels of serine proteinase activity, which could in principle target the PARs, have also been found in human colon tissue derived from individuals with ulcerative colitis (Hawkins et al., 1997) and inflammatory bowel syndrome (Cenac et al., 2007). Mast cell tryptase, which in humans can be released in the vicinity of sensory nerves, is another candidate enzyme that may regulate PAR₂ in vivo (Mirza et al., 1997; Molino et al., 1997a; Corvera et al., 1999). However, the action of tryptase seems to be restricted by the glycosylation site of PAR₂ in proximity to the cleavage-activation target of the enzyme (Compton et al., 2001; Compton et al., 2002a,b).

Although a number of other serine proteinases are known to trigger PAR₂ signalling (Steinhoff et al., 2005), the endogenous enzymes responsible for activating PAR₂ in settings other than the gastrointestinal and circulatory system, where trypsin-like enzymes can activate PAR₂ are unknown. Moreover, apart from the observations that neutrophil-released elastase and cathepsin G (Dulon et al., 2003), as well as Pseudomonas aeruginosa elastase (an elastolytic metalloproteinase contributing to bacterial pathogenicity) (Dulon et al., 2005), can dis-arm PAR₂, little is known about the proteinases that can inactivate either this receptor or other members of the PAR family.
Kallikrein-related peptidases (KLKs) compose a large family of secreted serine proteinases with trypic or chymotryptic activity (Borgoño et al., 2004; Borgoño and Diamandis, 2004). These enzymes, which share a high degree of genomic and protein sequence homology, are present in many mammalian species. The human kallikrein-related peptidases are abundantly expressed in groups in many tissues, often in a sex-steroid hormone-dependent manner, and they are up-regulated in disease states. For example, KLKs 5, 6, and 14 are found at increased levels in ovarian tumours or in serum and/or ascites fluid of ovarian cancer patients (Diamandis et al., 2000c; Kim et al., 2001; Tanimoto et al., 2001; Borgoño et al., 2003; Yousef et al., 2003e), as well as in the serum of breast cancer patients (Yousef et al., 2003e, Borgoño et al., 2003). KLKs are also highly expressed at sites of inflammation (Scarisbrick et al., 2002; Blaber et al., 2004).

However, despite their widespread expression in diseased tissues, the mechanisms whereby this enzyme family regulates cellular function are not clear. Proteins of the extracellular matrix, pro-urokinase-plasminogen activator, kininogens, growth factor precursors (and binding proteins), and other kallikrein-related peptidases are potential targets of KLK proteolysis during cancer progression (Frenette et al., 1997; Takayama et al., 2001a,b; Borgoño et al., 2004; Borgoño and Diamandis, 2004). Such targets may well explain some but by no means all of the physiological actions of kallikrein-related peptidases, particularly in the setting of cancer. PARs are also up-regulated in cancer and inflammation, and PARs have been proposed as substrates for KLKs (Borgoño and Diamandis, 2004). However, the ability of kallikrein-related peptidases to cleave and activate or disable PARs has not as yet been examined.

This Chapter deals with the major hypothesis of this thesis that the human KLK family of serine proteinases regulates signal transduction by cleaving the PARs. Using the active
enzyme preparations obtained from the work described in Chapter II, we tested our hypothesis using the following approaches:

1. A HPLC-mass spectral analysis of the cleavage products after incubation of KLK5, KLK6, and KLK14 with peptides corresponding to the N-terminal tethered ligand domains of PAR1, PAR2, and PAR4. In this section, proteolytically-generated fragments corresponding to putative receptor activation or inhibition sites were identified.

2. PAR-dependent calcium signalling responses in cell lines expressing PAR1, PAR2, and PAR4 and in isolated human platelets. Selection of the different cell systems allowed for evaluation of KLK action on PARs 1, 2 or 4 individually.

3. A PAR4-dependent rat and human platelet aggregation assay. As with the different cell culture systems above, selection of either human or rat platelets allowed for the evaluation of aggregation caused by KLKs either via PARs 1 and 4 (human) or via PAR4 (rat).

4. A vascular ring vasorelaxation assay using tissues from rats and both wild-type and PAR2-null mice. This part of the study made use of the PAR2-null mice to investigate the action of KLKs on PAR2 in whole tissue preparations.

For this part of the study we selected KLK14 as a “prototype” trypsin-like kallikrein, because of its particularly wide tissue distribution and high catalytic activity. The enzymatic properties of this protein are similar to the other two members of the family under study, KLKs 5 and 6, as well as to many other tryp tic kallikrein-related peptidases. We focused on the ability of KLK14 to regulate PAR2 in particular, because of the well recognized susceptibility of this PAR family member to trypsin-like serine proteinase activation. We also evaluated KLK14 for its ability to cleave/activate PAR1 and PAR4 and, for comparison, we determined whether KLKs 5 and 6 might also regulate PAR1, PAR2, and PAR4. To determine whether PARs from species other than humans might also be subject to KLK regulation, we used cell and tissue preparations
from rats and mice in addition to evaluating KLK action on human PAR1, PAR2, and PAR4. It should be noted that we chose not to include PAR3 in this study, as its signalling capability is still under question (Steinhoff et al., 2005). Rather, PAR3 seems to act as cofactor for thrombin signalling via PAR4 (Kahn et al., 1998; Nakanishi-Matsui et al., 2000; Sambrano et al., 2001).

### 3.2 Materials and Methods

#### 3.2.1 Reagents and peptides

All peptides were synthesized by standard solid phase methods by the Peptide Synthesis Facility at the Faculty of Medicine, University of Calgary. Peptide composition and purity were ascertained by HPLC analysis, amino acid analysis, and mass spectrometry. Stock solutions (about 1 mM) were prepared in 25 mM HEPES buffer, pH 7.4, and peptide concentrations were verified by quantitative amino acid analysis. Porcine trypsin (catalogue number T-7418; 14 900 Units/mg), phenylephrine, acetylcholine, L-NAME (the nitric-oxide synthase inhibitor, $N^\omega$-nitro-L-arginine methyl ester), and the calcium ionophore A23187 were from Sigma. High activity human thrombin (catalogue number 605195, lot B37722; 3 186 Units/mg) was from Calbiochem (Gibbstown, NJ, USA). The calcium indicator Fluo-3 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, USA), the sulfinpyrazone from Sigma (catalogue number S9509), and the PAR1 antagonist SCH 79797 from Tocris Bioscience (Ellisville, MO, USA). KLKs 5 and 14 were produced as previously described in Chapter II. The recombinant active KLK6 that was used for the experiments in this chapter was produced in a baculovirus/insect cell line system and activated by enterokinase, as previously described (Blaber et al., 2002; Yoon et al., 2007 and section 2.2.4). The protein was stored in 10 mM
sodium acetate, 0.01% Tween-20, pH 5.2. The agonists and antagonists utilized in the following experiments are summarized in Table 10.

### Table 10: List of symbols for agonists and antagonists.

<table>
<thead>
<tr>
<th>Agonist / Antagonist</th>
<th>Symbol used</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Ionophore</td>
<td>I</td>
<td>Control calcium response</td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td>Standard trypsic proteinase</td>
</tr>
<tr>
<td>KLK14 / KLK14, PAR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>□</td>
<td>KLK14 activity shown plus or minus pre-desensitization of PAR&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>KLK6</td>
<td></td>
<td>KLK6 activity shown plus or minus desensitization of other PARs</td>
</tr>
<tr>
<td>KLK5</td>
<td></td>
<td>KLK5 activity shown plus or minus desensitization of other PARs</td>
</tr>
<tr>
<td>SLIGRL-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>▽</td>
<td>Selective PAR&lt;sub&gt;2&lt;/sub&gt; agonist</td>
</tr>
<tr>
<td>TFLLR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>▼</td>
<td>Selective PAR&lt;sub&gt;1&lt;/sub&gt; agonist</td>
</tr>
<tr>
<td>SFLLR- NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>△</td>
<td>Non-selective PAR&lt;sub&gt;1&lt;/sub&gt;/PAR&lt;sub&gt;2&lt;/sub&gt; agonist, co-desensitizes both PARs 1 and 2</td>
</tr>
<tr>
<td>Thrombin, PAR&lt;sub&gt;1&lt;/sub&gt;</td>
<td>+</td>
<td>Activates PARs 1 and 4, but not PAR&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>KLK14, PAR&lt;sub&gt;1&lt;/sub&gt;</td>
<td>◀</td>
<td>KLK14 action in PAR&lt;sub&gt;2&lt;/sub&gt;-desensitized HEK cells</td>
</tr>
<tr>
<td>AYPGKF-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>△</td>
<td>Selective PAR&lt;sub&gt;4&lt;/sub&gt; agonist</td>
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<tr>
<td>SCH 79797</td>
<td>▲</td>
<td>PAR&lt;sub&gt;1&lt;/sub&gt; antagonist</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>□</td>
<td>Contractile alpha-1 adrenoceptor agonist</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>○</td>
<td>Endothelial muscarinic NO-releasing agonist</td>
</tr>
<tr>
<td>L-NAME</td>
<td>◄</td>
<td>Nitric Oxide Synthase (NOS) Inhibitor</td>
</tr>
</tbody>
</table>
3.2.2 Standardizing KLK enzyme activity

To facilitate direct comparisons between the different KLK preparations for their ability to activate or dis-arm the PARs, the concentration of each KLK preparation (KLKs 5, 6, and 14) was standardized for its enzymatic activity in terms of its “trypsin-like equivalents,” as follows. The enzymatic activity of kallikrein-related peptidases 5 and 6 was estimated essentially as described previously (Magklara et al., 2003; Michael et al., 2005), using the fluorescent synthetic substrate $t$-butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin (VPR-AMC) (Bachem Bioscience). In brief, kallikrein-related peptidase 5 was incubated at 37°C, in a microtiter plate, with the optimal activity assay buffer (100 mM Na$_2$HPO$_4$, 0.01% Tween-20, pH 8.0) and varying concentrations (within the linear range) of fluorescent substrates in a final volume of 100 µl. The initial rate of AMC release was measured on a Wallac Victor fluorometer (PerkinElmer Life Sciences) set at 355 nm for excitation and 460 nm for emission. The fluorescence values of enzyme-free reactions were used as negative controls and background fluorescence was subtracted from each value. All experiments were performed in triplicate. A standard curve with known concentrations of AMC was used to calculate the rate of product formation. Kinetic analysis was done by nonlinear regression analysis using the Enzyme Kinetics Module 1.1 (Sigma Plot, SSPS, Chicago, IL, USA). For KLK6, the assay was the same as for KLK5, but the buffer was changed to 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween-20, pH 7.3. The conditions for estimating the enzymatic activity of KLK14 were identical to those described for KLK5, except that $t$-butoxycarbonyl-Gln-Ala-Arg-7-Amino-4-methylcoumarin (QAR-AMC) was used as substrate (Borgoño et al., 2007c).

To standardize the activities of different KLK preparations, the activity of each KLK enzyme batch was expressed relative to the activity of a standard concentration of pure trypsin (5 nM or 2.5 Units/ml), measured using the same AMC substrate employed for assessing the
kallikrein-related peptidase activity. For molar calculations, 1 Unit/ml of trypsin enzyme was taken as 2 nM. Thus, the KLK concentrations shown as abscissa values in the figures are shown as Units of “trypsin-like equivalents of activity,” based on the above assays. This approach was used for a direct comparison between the proteinases, since each KLK preparation contained active enzyme along with a small (<10%) but variable proportion of inactive enzyme. Thus, any estimate of the enzyme EC₅₀ based on protein content alone would have been misleading. Therefore, the data shown in the figures reflect information specifically related to the active enzyme in each KLK preparation.

3.2.3 Mass spectral analysis of peptide proteolysis products

The following synthetic sequences derived from human and rat PAR₁, PAR₂, and PAR₄ were selected for the proteolysis studies (critical tethered ligand-activating sequences are underlined) (Tables 11 and 12): (a) hPAR₁, NATLDPRSFLRRNPNDKYE, (b) hPAR₂, N-acetyl-GTNRSSKGRSLIGKVDGTSHVTGKVGT-amide, (c) hPAR₄, GDDSTPSILPAPRGYGQV, (d) rPAR₂, GPNSKGRSLIGRLDTPYGGC (non-PAR₂ residues, YGGC, added for affinity column coupling and ¹²⁵I labeling), and (e) rPAR₄, LNESKSPDKPNPRGFPGK-P-amide. These peptides at a concentration of 100 µM were incubated with KLK5 (3.1 Units/ml), KLK6 (0.83 Units/ml), KLK14 (4.3 Units/ml) or trypsin (3.5 Units/ml) for varying times up to 30 min at 37°C. The incubation buffers were identical to the buffers used for the measurements of enzymatic activities. Reactions were terminated either by rapid freezing in liquid nitrogen or by the addition of 2 volumes of a “stop solution” comprising 50% acetonitrile and 0.1% trifluoroacetic acid in water. Samples were either subjected immediately to HPLC analysis, with collection of the peak fractions (UV Absorbance at 214 nm), or were stored at -80°C for further processing. The cleavage products were identified by three approaches as follows:
1. HPLC separation and isolation of the proteolysis products was followed by mass spectral MALDI identification of the peptide fragments in the quantified HPLC peaks. The HPLC analysis did not provide data for proteolysis products present in low abundance, so as not to be detected by the elution profile monitored by absorbance at 214 nm.

2. Combined liquid chromatography-mass spectral (LC-MS) analysis (Agilent HP 1100 Nanoflow System; Agilent Technologies Inc.) was followed by linear ion trap quadrupole analysis (Thermo Fisher Scientific Inc., Waltham, MA, USA) of the entire proteolysis mixture without prior HPLC separation. For this second approach, the relative abundance of the LC-separated constituents was not determined.

3. Matrix-assisted laser desorption ionization (MALDI)-mass spectrometry of the complex hydrolysis reaction mixture was performed with computer-assisted deconvolution of the mass spectra. Like the LC-MS approach, this method did not provide information about the relative abundance of the peptides detected.

Analyses were done either at the proteomic facilities of the Samuel Lunenfeld Research Institute (Mount Sinai Hospital, Toronto, ON, Canada) or at the Southern Alberta Mass Spectrometry Facility at the University of Calgary, Faculty of Medicine (Calgary, AB, Canada).

3.2.4 Cell culture and PAR-expressing cell lines

Kirsten virus-transformed normal rat kidney (KNRK) epithelial cell lines expressing either wild-type rat PAR2 (KNRKr-PAR2) or the trypsin-resistant rat PAR2(R36A) variant were validated previously for studies of receptor activation (Al-Ani and Hollenberg, 2003) and were, therefore, used to evaluate the PAR2-activating properties of the kallikrein-related peptidases by the methods described previously (Kawabata et al., 1999; Al-Ani and Hollenberg, 2003).
Human embryonic kidney cells (HEK-293) that express the SV40 T-antigen were kindly provided by Dr. Jonathan Lytton (University of Calgary, Faculty of Medicine, Calgary, AB, Canada). The HEK-293 cells that constitutively express both PAR₁ and PAR₂ were grown as for the KNRK cells, but in the absence of geneticin, as outlined for the PAR-mediated calcium signalling procedure described previously (Kawabata et al., 1999).

A rat PAR₄-expressing HEK-293 cell line HEK-rPAR₄ was generated from a receptor clone kindly provided by Dr. W. A. Hoogerwerf (Galveston, TX, USA; Hoogerwerf et al., 2002). In brief, using the rat PAR₄ clone, a N-terminal Myc and C-terminal herpes simplex virus tag were added by PCR employing the primers: forward: 5’-GAGCAGAAGCTGATCAGCGAAGAGGACCTGCCGGGAATGCCAGACGCC-3’, and reverse: 5’-ATCTCGAGTCAGCAGTCCTCGGGGTCCTCGGGGGCCAGCTCGGGCTGCAGAAGTGTAGAGGAGCAAATG-3’, using standard techniques. The modified cDNA was then sub-cloned into the vector pcDNA3.1-Igκ neo (Cottrell et al., 2004) to yield the expression vector pcDNA3.1-Igκ mycPAR4HSV. This vector was used to transfect the HEKFLP cell line (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guidelines. After two days, the cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 200 µg/ml G418 sulfate and 100 µg/ml Zeocin for 3 weeks. Individual foci were expanded and assessed for expression by immunofluorescence. After selection, the rat PAR₄-expressing HEK-293 cells (HEK-rPAR₄) were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 100 µg/ml G418 sulfate, 100 µg/ml Zeocin. A vector control cell line was similarly engineered and propagated. All cells were sub-cultured by dissociation in an isotonic EDTA/saline solution, pH 7.4, without the use of trypsin, to avoid cleavage of PARs.
3.2.5 Immunohistochemical detection of PAR2 cleavage/activation

To assess morphologically the ability of the KLKs to cause activation or “dis-arming” of PAR2, we made use of a rat PAR2 expression system (receptor expressed in the KNRK cells) along with two rat PAR2-targeted antisera that recognize either the entire cleavage-activation sequence (B5) (Al-Ani and Hollenberg, 2003) or only the pre-cleavage sequence (SLAW-A) (Compton et al., 2001; Al-Ani and Hollenberg, 2003; Wang et al., 2003). The use of these two antisera to document receptor activation or dis-arming has been validated for rat PAR2 both with cells grown in vitro and in tissues monitored in vivo (Compton et al., 2001; Al-Ani et al., 2002; Al-Ani and Hollenberg, 2003; Wang et al., 2003). Unfortunately a comparable validation of similar antisera to monitor the activation/dis-arming of human PAR2 has not yet succeeded.

With this approach, activation of the receptor, without a downstream cleavage that would remove the tethered ligand entirely, results in disappearance of reactivity with the SLAW-A antibody but retention of reactivity with B5. Alternatively, cleavage of the receptor downstream of the tethered ligand sequence abolishes the reactivity of the cells with both the B5 and SLAW-A antisera. Polyclonal rabbit anti-PAR2 antisera (B5 and SLAW-A) were raised in rabbits and used as described elsewhere (Kong et al., 1997; Compton et al., 2001; Al-Ani and Hollenberg, 2003). The antibodies were generated against peptides that corresponded to sequences representing the potentially antigenic epitopes either in the pre-cleavage domain of wild-type rat PAR2 (for SLAW-A, SLAWLLGGPNSKGR) or in the tethered ligand cleavage/activation site (denoted by “/”) of rat PAR2 (for B5, GPNSKGR/SLIGRLDTP).

As mentioned, one or both of these epitopes can potentially be lost from the cell surface upon cleavage/activation of the receptor by proteinases. The removal of the N-terminal antigenic determinants by KLKs or by trypsin was assessed by monitoring the decrease of cell surface reactivity with the SLAW-A and B5 antibodies (Compton et al., 2001; Al-Ani et al.,
Neither the B5 nor the SLAW-A antibodies reacted with KNRK cells transfected with vector alone. Furthermore, the reactivity of both antibodies with PAR$_2$-expressing KNRK cells is abolished by pre-absorption with the immunizing peptide (Compton et al., 2001; Al-Ani et al., 2002).

To monitor the ability of KLKs 5, 6, and 14 to cleave PAR$_2$ at or beyond the tethered ligand activation site, KNRK-rPAR$_2$ cell suspensions were treated or not for 5 min at room temperature with enzyme concentrations that had been documented to activate rat PAR$_2$ in the calcium signalling assay. The effects of the KLKs were compared with the actions of trypsin (20 Units/ml; about 40 nM). For immunocytochemical morphometric analysis, enzyme-treated cells were spun onto a glass microscope slide, using a Shandon cytospin apparatus (Shandon Scientific, Cheshire, UK), washed with PBS (5 sec), fixed in 95% ethanol (15 min), washed with PBS (twice for 5 sec), and permeabilized with 0.2% Triton-X in PBS (5 min). Next, the cells were treated with a solution of 3% bovine serum albumin and 0.05% Tween-20 in PBS (5 min), washed with PBS (5 sec), and incubated with either the SLAW-A (1:250 final dilution) or B5 (1:500 final dilution) antibodies in antibody diluting buffer (DAKO Diagnostics Canada Inc., Mississauga, ON, Canada) for 1 h in a humidity chamber. As a control, one set of cells was treated with a mixture of antisera pre-incubated with the immunizing peptide (SLAWLLGGPNSKGRGGGYGGC for SLAW-A; GPNSKGRSLIGRLDTPYG GC for B5; each 1 µg/ml) instead of antibody alone. Finally, the cells were washed with PBS (three times for 5 sec), incubated with anti-rabbit IgG Cy 3 conjugate (Sigma) (30 min), washed with PBS (5 sec), air-dried, and mounted onto slides with 90% glycerol, 10% Tris (1 M, pH 8) mounting medium. The fluorescence was visualized using a Leica microscope (Cambridge, UK), and images were analyzed using the software Image J (http://rsb.info.nih.gov/ij).
For experiments done on three separate occasions with independently cultured cell samples, images of five or more different representative fields of view were recorded. The fluorescence intensity was then measured at five different points along the cell surface in each of the five or more images, and averages were taken. The ratio of the fluorescence values resulting after proteolytic cleavage by an enzyme with respect to the fluorescence of non-treated cells was used to determine the percentage of receptors not cleaved. The percent cleavage was determined by subtracting the above number from 100. The numbers reported in this study correspond to the means and s.e.m. of measurements obtained from the three different experiments. A control cell population of KNRK cells expressing wtPAR2 regularly scored >80–90% positive; vector-transfected cells or stained preparations in which the antisera had been pre-absorbed with the immunizing peptides were routinely negative, as described previously (Compton et al., 2001; Al-Ani et al., 2002; Al-Ani and Hollenberg, 2003).

In summary, disappearance of SLAW-A reactivity, with retention of B5 reactivity, reflected cell activation by cleavage at the R/S tethered ligand cleavage-activation site of PAR2. Alternatively, disappearance of cellular reactivity toward the B5 antiserum (the SLAW-A reactivity would also be absent) indicated dis-arming of the receptor by cleavage downstream from the tethered ligand sequence, as observed previously for neutrophil elastase (Dulon et al., 2003). The quantitative morphometric analysis used to quantify receptor activation/dis-arming has been validated previously for both cultured cells and intact tissues (Compton et al., 2001; Al-Ani and Hollenberg, 2003; Dulon et al., 2003; Wang et al., 2003).

### 3.2.6 Measurements of PAR-regulated calcium signalling

Cell lines (grown to about 85% confluency and disaggregated with calcium-free isotonic phosphate-buffered saline containing 0.2 mM EDTA) and rat and human platelets (harvested
from platelet-rich anticoagulated plasma) were incubated for 25 min at room temperature with Fluo-3 acetoxyethyl ester (final concentration, 22 µM) along with 0.4 mM sulfinpyrazone. Cells were washed twice by centrifugation (using the following buffer without calcium) and resuspended in the calcium signalling buffer: 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 20 mM HEPES, 10 mM dextrose, and 0.25 mM sulfinpyrazone, pH 7.4. Fluorescence was measured at 24°C with an excitation wavelength of 480 nm and an emission recorded at 530 nm using an Aminco Bowman Series 2 Luminescence spectrometer (ThermoSpectronic Model FA354, Spectronic Unicam, Rochester, NY, USA). The fluorescence signals caused by the addition of test agonists (trypsin, KLKs, or PAR-activating peptide, added to 2 ml of a cell suspension of about 3 x 10⁵ cells/ml) were compared with the fluorescence peak height yielded by replicate cell suspensions treated with 2 µM calcium ionophore A23187. This concentration of A23187 was at the plateau of its concentration-response curve for a fluorescence response in Fluo-3-loaded cultured cells. Under these conditions, the calculated values for intracellular calcium in the HEK-293 and KNRK cells were ~30 nM under basal conditions and about 340 nM upon exposure to A23187 (Kao et al., 1989; Minta et al., 1989). Previous work (Kawabata et al., 1999; Compton et al., 2000) has shown that the fluorescence response of a cell preparation, as a percentage relative to the signal generated by 2 µM A23187, is a valid reference standard for the comparative determination of calcium signals for all PAR agonists. The cellular calcium response was therefore recorded as a percentage of the response generated by 2 µM ionophore A23187 (% A23187). Concentration-effect curves for KLK14 and KLK6 were obtained by measuring the calcium signals generated by increasing enzyme concentrations in replicate cell suspensions (2 ml). Values at each enzyme concentration represent the averages (± s.e.m.: error bars above average values) of measurements done with at least three cell replicates derived from three or more independently grown cultures of PAR-expressing cells.
3.2.7 Receptor desensitization protocol

To monitor the cellular response to an individual PAR in the HEK-293 cells that express multiple PARs (PAR₁ and PAR₂ in background HEK-293 cells; PAR₁, PAR₂, and PAR₄ in HEK-rPAR₄ cells), we made use of a selective receptor desensitization protocol (Kawabata et al., 1999). Cells were exposed to a PAR agonist at a supramaximal concentration (to fully desensitize a selected PAR) or vehicle (control) and were then challenged with a second agonist after a 5-10-min interval, sufficient for the re-filling of the intracellular calcium stores (e.g. see Kawabata et al., 1999). To assess the ability of KLKs 5, 6, and 14 to activate one or both of PAR₁ and PAR₂, the HEK-293 cells were first desensitized by exposure to either the PAR₁-AP, TFLLR-NH₂, or the PAR₂-AP, SLIGRL-NH₂ (or both), followed by the addition of KLKs 5, 6 or 14. The persistence of a calcium signal in response to the KLKs after desensitization of PAR₁ by TFLLR-NH₂ demonstrated the activation of PAR₂ by the proteinase, and the persistence of a calcium signal after desensitization caused by the PAR₂-AP, SLIGRL-NH₂, indicated an activation of PAR₁. Furthermore, the complete disappearance of a KLK-induced calcium signal after pre-desensitizing the HEK-293 cells with TFLLR-NH₂ and SLIGRL-NH₂ in combination (100 µM each) or with 200 µM of the PAR non-selective agonist SFLLR-NH₂ would serve to indicate that the calcium signal caused by the proteinase did not result from the activation of a non-PAR mechanism.

3.2.8 Evaluation of receptor dis-arming/inhibition (calcium signalling and desensitization)

Proteolytic removal of the tethered ligand by cleavage downstream from the activation domain can dis-arm a PAR, so as to inhibit its proteolytic activation but still leave the cell sensitive to activation by a PAR-activating peptide (Kawabata et al., 1999; Dulon et al., 2003,
In this process the proteinase amputates the receptor-activating sequence but may possibly also bind tightly in a non-catalytic mode to the receptor cleavage site, thereby inhibiting activation by other proteinases. To determine whether KLK14 dis-arms/inhibits PAR₁ for activation by thrombin, we exposed wild-type HEK-293 cells for 10 min to increasing concentrations of KLK14, starting with concentrations determined to result in a minimal activation of PAR₁. In the continued presence of KLK14, the cells were then challenged either with a PAR₁-activating concentration of thrombin (0.5 Units/ml; 5 nM) or with a PAR₁-activating concentration of TFLLR-NH₂ (5 µM). The thrombin-induced and peptide-induced calcium signals measured after KLK14 exposure were compared (% control) with the “control” calcium signal caused by these agonists in the absence of KLK exposure in the same cell preparation. The diminished “residual” PAR₁ response to thrombin represents both dis-arming and desensitization. As a variation on this protocol, cells first pre-treated or not with KLK14 for 10 min were washed free of enzyme and resuspended in calcium assay buffer for the measurement of thrombin-activated and peptide-activated calcium signalling. This protocol ensured that the residual KLK14 in the incubation medium did not block or otherwise inhibit thrombin action. Again a reduced thrombin response in the washed, KLK14-treated cells, with retention of the signal caused by TFLLR-NH₂, reflected a dis-arming of PAR₁. Alternatively, the diminished response to the peptide after KLK14 treatment represents only receptor desensitization and not dis-arming (Kawabata et al., 1999). In the same cell preparation, the PAR₁ calcium signal triggered by KLK14 itself (monitored after desensitization of PAR₂ by prior treatment with SLIGRL-NH₂) was also measured. Because of the complexity of this procedure (which requires the use of non-desensitizing / non-activating concentrations of KLK14 to observe receptor dis-arming), it was not possible to do comparable experiments to
measure with confidence the dis-arming by KLK14 of either PAR2 or PAR4 for activation by either trypsin (PAR2 and PAR4) or thrombin (PAR4).

### 3.2.9 Platelet isolation and aggregation assay

Venous anticoagulated blood (0.5 ml of 3.4% w/v trisodium citrate per 10 ml of blood) was collected from human male volunteers and from male Sprague-Dawley rats (200-300 g, inferior vena cava puncture) by institutionally approved protocols. A platelet-rich plasma suspension was obtained (Wallace and Woodman, 1995) by centrifugation (900 rpm; 150 x g\text{max}) at room temperature for 15 min (r\text{max} = 170 mm). The platelet-rich plasma supernatant was withdrawn for the preparation of washed platelets. To prepare washed platelets, the platelet suspension was supplemented with 0.8 \(\mu\)M prostaglandin I2 (300 ng/ml), harvested by centrifugation (1800 rpm; 620 x g\text{max} for 10 min at room temperature), and resuspended in prostaglandin I2 (0.8 \(\mu\)M)-supplemented calcium-free Tyrode’s buffer, pH 7.4, of the following composition: NaCl (136 mM), KCl (3 mM), NaHCO3 (12 mM), NaH2PO4 (0.4 mM), MgCl2 (1 mM), and glucose (6 mM). Platelets were again collected by centrifugation (1400 rpm; 370 x g\text{max} for 10 min at room temperature) and resuspended in calcium-free Tyrode’s buffer. For use in the aggregation assay, the suspension was then made up to 1 mM CaCl2 and supplemented with indomethacin (10 \(\mu\)g/ml; 28 \(\mu\)M). Platelets to be used for the calcium signalling experiments were obtained as described above and harvested by centrifugation from the platelet-rich plasma fraction obtained immediately after the first centrifugation step used to process the citrate-anticoagulated blood. The platelet pellet obtained from the platelet-rich plasma was resuspended in the isotonic Fluo-3-containing HEPES buffer, pH 7.4, described above, except that calcium was omitted from the solution for the Fluo-3 uptake procedure and the two subsequent washes. For aggregation studies, the resulting washed platelet suspension in
the indomethacin-containing calcium-replete buffer was allowed to stand at room temperature for 1 h to allow for degradation of residual prostaglandin I₂ before use as 0.4-ml aliquots (2-3 x 10⁸ platelets/ml) in calcium-replete (1 mM) Tyrode’s buffer, pH 7.4. Light transmission was monitored with a dual channel aggregometer (Payton Scientific, Buffalo, NY, USA). Agonists were added directly to the 0.4-ml suspension at 37°C, and aggregation was quantified as a percentage (% thrombin) of the maximal aggregation (i.e. maximal increase in light transmission) caused by 1 Unit/ml (10 nM) human plasma thrombin. For calculating the molar concentration of thrombin, 1 Unit/ml was taken as equivalent to 10 nM.

3.2.10 Aorta relaxation

The endothelium-intact rat and mouse aortic ring assay used to monitor the responses to the PAR-activating peptides and the kallikrein-related peptidases was essentially the same as that described previously for measuring the actions of PAR₂-activating peptides (Al-Ani et al., 1995; Hollenberg et al., 1997). In brief, male Sprague-Dawley rats (250-300 g) or male C57/BL6 mice (either wild-type or PAR₂-null, about 50-70 g), cared for in accordance with the Guidelines of the Canadian Council on Animal Care, were killed by cervical dislocation. Clot-free portions of aorta were dissected free of adherent connective tissue, and endothelium-intact rings (approx. 2 mm length x 2 mm outer diameter) were cut for use in the bioassay. Aortic rings were equilibrated at 1 g resting tension for 1 h at 37°C in a gassed (5% CO₂, 95% O₂) modified Krebs-Henseleit buffer, pH 7.4, of the following composition: NaCl (118 mM), KCl (4.7 mM), CaCl₂ (2.5 mM), MgCl₂ (1.2 mM), NaHCO₃ (25 mM), KH₂PO₄ (1.2 mM), and glucose (10 mM). The relaxant actions of trypsin, the KLKs or the PAR-activating peptides (about 2 µM SLIGRL-NH₂ or 5 µM TFLLR-NH₂) were measured in endothelial intact aortic rings that were pre-contracted with 1 µM phenylephrine. A relaxant response to 10 µM
acetylcholine (a 60–95% reduction of the tension generated by 1 µM phenylephrine) was taken as a positive index for an intact endothelium. To assess the contribution of the endothelium to the relaxation response, endothelium-free preparations were used, in which the endothelium was destroyed by rolling the aortic rings against a thin wire. The absence of endothelium was verified by observing an absence of a relaxant response to 10 µM acetylcholine. Peptides were added directly to the organ bath (4-ml cuvette), and the development of tension and subsequent relaxation exhibited by the rings was monitored using either Grass or Statham force-displacement transducers. When present, L-NAME (0.1 mM) was added to the organ bath 10 min prior to the addition of other reagents.

3.3 Results

3.3.1 Analysis of synthetic tethered ligand peptide proteolysis by HPLC and MALDI

Because our study dealt with KLKs of human origin, we elected first to evaluate their ability to cleave the peptides corresponding to regions of the N-terminal domains of human PAR₁, PAR₂, and PAR₄, with a focus on KLK14 as a prototype kallikrein-related peptidase (Tables 11 and 12) and PAR₂ as its main target. Cleavage by KLKs 5 and 6 was also studied, but the limited amounts of these enzymes available for our study did not permit an extensive analysis as with KLK14. HPLC separation followed by mass spectral identification of the proteolysis fragments in the peaks generated by KLK14 from each of the synthetic tethered ligand sequences of hPAR₁, PAR₂, and PAR₄ (Figure 13A) showed that the expected receptor-activating sequences (SFLLRN… for PAR₁; SLIGKV… for PAR₂; and GYPGQV… for PAR₄) were released by cleavage at the R/S (PAR₁ and PAR₂) or R/G (PAR₄) bonds (Tables 11 and 12; Figure 13A). The degree of peptide cleavage demonstrated by the relative peak areas of the
HPLC analysis indicated that, although there was significant cleavage of the hPAR2- and hPAR4-derived peptides (≥50%), only a modest cleavage (about 20%) of the hPAR1 peptide had occurred (Figure 13A, tracings A-C). The HPLC analysis also demonstrated appreciable KLK14 cleavage of the human PAR2 sequence at the arginine upstream of the tethered ligand sequence (TNR/SSK). In addition, identification of the PAR1 cleavage sequence, NATLDPRSFLLR (Figure 13A, tracing A), indicated that KLK14 cleaved within the tethered ligand sequence of hPAR1 at a site (LLR/NPN) that would “dis-arm” the receptor. This cleavage would remove the tethered ligand sequence from the remainder of the receptor, thereby preventing its activation by thrombin. HPLC-mass spectral analysis also demonstrated KLK14 cleavage of the comparable rat PAR2 and PAR4 N-terminal peptide sequences at the R/S and R/G sites that would generate the receptor-activating sequences (Figure 13A, tracings D and E). Cleavage of a peptide corresponding to the N-terminal of rat PAR1 was not studied. The time course of KLK14 cleavage of the human and rat PAR2 peptides revealed that at 30 min the receptor-activating peptides (SLIGKV… or SLIGRL…) represented the major proteolysis products generated from both peptides. For the human PAR2 sequence, this peptide (SLIGKV…) represented 14% of the amount of the intact peptide at 5 min, 39% at 10 min, and 120% of the remaining amount of intact peptide at 30 min (Figure 13A, tracing B). The rate of KLK14 hydrolysis of the rat PAR2 peptide was more rapid, with the amount of receptor-activating peptide released at 30 min (SLIGRL…) representing about 250% of the remaining intact peptide (Figure 13A, tracing D). Thus, the key R/S bond that reveals the receptor-activating sequence represents the main KLK14 target in both human and rat PAR2. These results paralleled closely the cleavage products yielded by trypsin-mediated hydrolysis of the PAR-derived peptides (not shown). In this regard, the cleavage by trypsin to yield the human and rat PAR-activating peptide sequences was greater in extent than that of KLK14.
A comparable HPLC-mass spectral analysis of the cleavage products generated by KLK5 and KLK6 from the PAR-derived peptides yielded results consistent with those shown for KLK14 in Figure 13A, with the exception of the hydrolysis of the PAR_4 peptides. Peptide hydrolysis by these two KLKs released significant amounts of the receptor-activating peptide for human and rat PAR_2 and small amounts of the hPAR_1-activating peptide (Figure 13B for KLK6; data not shown for KLK5). The peptide which was generated by KLK14 suggesting a disarming cleavage of PAR_1 (Figure 13A, tracing A, 1st peak on the right) was not observed for the cleavage of the hPAR_1 peptide by either KLK5 or KLK6. The human PAR_4 peptide was cleaved by both KLKs 5 and 6 to yield a receptor-activating peptide (about 10% of the uncleaved peptide at 30 min), but no release by KLKs 5 and 6 of a PAR_4-activating peptide was detected by HPLC analysis from the rat-derived PAR_4 sequence (Figure 13B for KLK6; data not shown for KLK5), despite its detection by the LC-MS approach (see next section). In summary, we found that like KLK14, both KLK5 and KLK6 could cleave the human PAR_1, PAR_2, and PAR_4 synthetic peptide sequences to generate receptor activating peptides, with a predominant cleavage of the hPAR_2 peptide compared with PAR_1 and PAR_4. Although KLK5 and KLK6 cleavage of the rat PAR_2-derived sequence also yielded significant amounts of a receptor-activating peptide, the rPAR_4 sequence did not yield amounts of a receptor-activating peptide that could be detected by HPLC analysis.
Figure 13: HPLC separation and mass spectral analysis of PAR cleavage products generated by KLK14 (A) and KLK6 (B). Peptides representing the tethered ligand sequences of human PARs 1, 2, and 4 (panels A to C) and rat PARs 2 and 4 (Panels D and E) were subjected to proteolysis over a 30 min time period as described in Materials and Methods. The peptides in the quenched reaction product were separated by HPLC analysis, and peptides in the peak fractions (Absorbance: E 214) were collected and subjected to mass spectral analysis. The sequences of the peptides deduced from the mass spectral data are indicated next to each peak. Underlined amino acid residues represent the key receptor-activating tethered ligand sequences. The double arrows in each panel represent 0.01 absorbance units.
3.3.2 Analysis of peptide proteolysis by LC-MS or MALDI

In addition to the approach described above, we subjected the entire proteolysis reaction mixture to LC-MS and MALDI analysis without prior HPLC separation of the cleaved peptides. This approach enabled us to detect minor cleavages that would have been missed by the HPLC separation approach but did not permit an analysis of the relative abundance of the cleavage products identified. Cleavage of rat PAR₁ was not studied. This analysis (Table 11) confirmed the release by KLKs 5, 6, and 14 of the tethered ligand sequences from human PARs 1, 2, and 4 and from rat PAR₂. A minor cleavage of rat PAR₄ to yield its receptor-activating sequence was also suggested (Table 11). In addition, the analysis confirmed the cleavage of the hPAR₁ sequence at the LLR/NP bond that would dis-arm the receptor. Other cleavages in the PAR peptides were also detected at predicted tryptic sites of serine proteinase proteolysis (at a lysine either within or C-terminal to the tethered ligand sequence of human PAR₂ or at a lysine C-terminal to the tethered ligand of rat PAR₄, to yield the peptide sequence LNESKSPDKPNPRGFPGBK). This second cleavage would not be expected, given the inability of trypsin to cleave if a proline residue is on the carboxyl side of the cleavage site, because of the proline amino group. Although these peptides found by the LC-MS or MALDI approaches must be presumed to result from minor sites of cleavage (the majority of them were not detected by the HPLC analysis; see above), they would in principle also dis-arm the two PARs. In general, cleavage of the PAR peptide sequences by the KLKs reflected comparable cleavages caused by trypsin, especially in terms of revealing the tethered ligand sequences of the PARs. Added to the expected cleavages at arginines or lysines, the LC-MS and MALDI analyses pointed to unlikely chymotryptic sites of cleavage caused by the KLKs, e.g. at the SF/LL bond of human PAR₁ or the GY/PG site in hPAR₄ by KLK14 or KLK6 (Tables 11 and 12). These cleavages may be due to the recognized minor chymotryptic activity of KLKs, such as KLK14.
(Felber et al., 2005) and KLK6 (Sharma et al., 2008). Other unusual sites of cleavage were also suggested by the LC-MS and MALDI analyses. However, the expected chymotryptic and other peptides were not detected by HPLC-MS analysis (Figure 13 as above). Thus, KLKs 5, 6, and 14 can cleave synthetic peptides representing the N-terminal sequences of PAR₁, PAR₂, and PAR₄ to expose their receptor-activating moieties. These sequences, when unmasked by the KLKs in vivo, would lead to activation of the receptors in intact cells. In addition, these three proteinases can also cleave at sites that would excise the tethered ligand sequences, thereby disarming these PARs. The major sites of KLK cleavage shown by the HPLC peaks in Figure 13, as well as other minor cleavages detected by the LC-MS and MALDI analyses are summarized in Table 12, where both the major (bold upward arrows, detected by HPLC-MS analysis) and minor sites (downward arrows, detected by LC-MS/MALDI analysis) of cleavage are indicated. A possibility that cannot be ruled out is that the unexpected cleavage products detected by the LC-MS/MALDI analysis of the entire hydrolysis mixture may have resulted from peptide fragmentation because of the mass spectral procedure itself or, although unlikely, because of the presence of undetected non-KLK proteinase contaminants in the KLK preparations. The potential physiological impact of the minor sites of cleavage is difficult to assess.
Table 11: KLK-generated proteolytic products of PAR-derived synthetic peptides identified by mass spectrometry without HPLC separation.

<table>
<thead>
<tr>
<th>PAR</th>
<th>Peptide Sequence</th>
<th>Trypsin</th>
<th>KLK14</th>
<th>KLK5</th>
<th>KLK6</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPAR1 (NATLDPRSFLLRNPNDKYE)</td>
<td>NATLDPR—SFLLRNPNDKYE</td>
<td>NATLDPR—SFLLRNPNDKYE</td>
<td>NATLDPRSF—LLRNPNNDKYE</td>
<td>NATLDPRSFLLRNPNNDKYE</td>
<td>NATLDPRSFLLRNPNNDKYE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NATLDPRSF—LLRNPNNDKYE</td>
<td>NATLDPRSFLLRNPNNDKYE</td>
<td>NATLDPRSFLLRNPNNDKYE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NATLDPRSFSLLRNPNNDKYE</td>
<td>NATLDPRSFSLLRNPNNDKYE</td>
<td>NATLDPRSFSLLRNPNNDKYE</td>
</tr>
<tr>
<td>KLK14</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
</tr>
<tr>
<td>KLK5</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
</tr>
<tr>
<td>KLK6</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
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<td>GDNSSKKGR—SLIGKVDGTVSHVTRGKV</td>
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Table 11 (Continued)

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rPAR4 (LNESKSPDKPNPRGFPGKP-Amide)

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<td>LNESKSPDKPNPRGFPGK—P</td>
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</table>

\(^1\)Footnote: Synthetic peptides representing the tethered ligand sequences of human and rat PARs (bold type in each section) were incubated with Trypsin (3.5 U/ml), KLK14 (4.3 U/ml), KLK5 (3.1 U/ml), and KLK6 (0.83 U/ml). Cleavage products in the unfractionated reaction mixture were identified by LTQ and/or MALDI mass spectrometry. Proteolytically generated sequences that represent the active tethered ligands for PARs 1, 2, and 4 are shown in underlined bold italics.
Table 12: Scheme of proteolysis of PAR tethered ligand sequences by human KLKs 5, 6, and 14\(^1\).

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<th>Trypsin</th>
<th>hK14</th>
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\(^1\) Footnote: Major sites of cleavage confidently detected by HPLC-Mass Spectral and by LC-MS analysis are denoted by BOLD UPWARD arrows; minor sites of cleavage detected only by LC-MS/MALDI analysis of the complex peptide hydrolysis mixture are denoted by downward arrows. The tethered ligands for PARs 1, 2, and 4 are underlined.

3.3.3 Evaluation of KLK-mediated PAR\(_2\) cleavage in intact cells

We have found previously that the observed cleavage of model PAR peptides by trypsin or thrombin may or may not reflect the ability of the enzymes to cleave/activate or disarm/inhibit the receptors in intact cells (Al-Ani and Hollenberg, 2003). Thus, to assess whether KLK14 can cleave intact PAR\(_2\) at the cell surface, we used an immunohistochemical approach to monitor PAR\(_2\) cleavage in intact rat PAR\(_2\)-expressing KNRK cells (KNRK-rPAR\(_2\)), as outlined under Materials and Methods. The expressed rat receptor was used for our study because of our previous success with the analysis of trypsin cleavage of this receptor in intact cells and intact tissues (Al-Ani and Hollenberg, 2003; Wang et al., 2003). The analysis depends upon the reactivity of two targeted antisera (B5 and SLAW-A) with two distinct domains of rat PAR\(_2\) (Al-Ani and Hollenberg, 2003). The B5 antiserum detects the cleavage-activation
sequence of PAR₂, whereas the SLAW-A antiserum detects only the pre-cleavage sequence. When KNRK-rPAR₂ cells were incubated with KLK14 under conditions that would result in calcium signalling (see below), morphometric analysis of the immunoreactive cells revealed that there was a loss of reactivity with the SLAW-A antiserum (a reduction in signal relative to untreated cells of 80 ± 3%, mean ± s.e.m., n = 5) but retention of reactivity toward B5 (Figure 14). Trypsin also removed the SLAW-A epitope (reduction of SLAW-A fluorescence signal by 84 ± 2% relative to control cells) (Figure 14). These data indicated that cleavage at the GR/SL bond of PAR₂ had occurred, removing the epitope(s) reacting with the SLAW-A antiserum, but cleavage at other downstream sites (either tryptic or chymotryptic), which would have released the epitope(s) reacting with the B5 antiserum, had evidently not occurred. Treatment of the KNRK-rPAR₂ cells with KLK6 produced results comparable with those obtained with KLK14 (a reduction in the SLAW-A signal by 69 ± 5%, relative to control cells). KLK5 was also able to remove the epitope(s) detected by the SLAW-A antiserum but to a lower degree (a reduction in signal of 28 ± 4%, mean ± s.e.m., n = 5) than was observed for KLK6 and KLK14 (Figure 14). As was evident from examining multiple microscopic fields like those shown in Figure 14, neither KLK6 nor KLK5 was able to decrease the reactivity of the B5 antiserum with the cells. The results indicated that, as for KLK14, the tethered ligand had been proteolytically revealed but that appreciable cleavage downstream of the tethered ligand sequence had not occurred.
Figure 14: KLK-mediated removal of the pre-cleavage sequence of rat PAR2 in intact cells. KNRK cells expressing rat PAR2 were treated or not with either trypsin or KLKs 5, 6, and 14 under conditions that would have produced a calcium signal. The presence or absence of the pre-cleavage epitope detected by the SLAW-A antibody (left-hand panels) and the tethered ligand portion of PAR2 detected by the B5 antibody (right-hand panels) were then monitored, as outlined in Materials and Methods. Pre-absorption of the SLAW-A and B5 antisera with the immunizing peptides eliminated the PAR2-specific immunoreactivity in enzyme-untreated cells (bottom two panels). The micrograph is representative of 5 or more randomly selected optical fields used for the morphometric analysis calculations that were based on three independently conducted experiments, as outlined in Materials and Methods.
3.3.4 Calcium signalling triggered by KLK-mediated activation of PAR$_1$ and PAR$_2$

To determine whether KLK14 cleaves and activates PAR$_2$, we measured its effects on calcium signalling by both the human and rat receptors expressed in intact cells. We first tested KNRK-rPAR$_2$ cells that were used for the immunohistochemical monitoring of PAR$_2$ activation. These cells express only rat PAR$_2$ and not the other PARs. In the KNRK-rPAR$_2$ cells, KLKs 5, 6, and 14 were all able to generate a calcium signal comparable with that of trypsin (Figure 15A, tracing A) but did not produce a signal in the non-transfected KNRK cells at the same enzyme concentrations (not shown). Furthermore, pre-desensitizing the PAR$_2$-generated calcium signal in the KNRK-rPAR$_2$ cells by pre-exposure to a high concentration of the PAR$_2$ agonist, SLIGRL-NH$_2$ (50 µM), prevented the subsequent KLK-triggered calcium signal (Figure 15A, tracing B). Finally, in the KNRK cell line expressing the rat PAR$_2$(R$^{36}$A) mutant receptor, in which the R$^{36}$A mutation abrogates trypsin-mediated (but not SLIGRL-NH$_2$-triggered) activation of PAR$_2$ (Figure 15A, tracing C, first result on the left) (Al-Ani and Hollenberg, 2003), none of KLKs 5, 6 or 14 caused a calcium signal (Figure 15A, tracing C, right-hand results) at concentrations that did so in the wild-type KNRK-rPAR$_2$ cells (Figure 15A, tracing A, right-hand results). As expected, the selective PAR$_2$-activating peptide, SLIGRL-NH$_2$, did elicit a calcium signal in the PAR$_2$(R$^{36}$A) cells (Figure 15A, tracing C, right-hand tracings). Thus, kallikrein-related peptidases 5, 6, and 14 activate rat PAR$_2$ at the same Arg$^{36}$/Ser$^{37}$ site as trypsin to expose the tethered ligand sequence. The concentration-effect curve for KLK14-mediated activation of PAR$_2$ calcium signalling was compared with the curves for KLK6 and trypsin in the KNRK-rPAR$_2$ cells (Figure 15B). The relative potencies (inverse of the EC$_{50}$ values) of the three enzymes were as follows: trypsin $>$ KLK6 $>$ KLK14, with the potency of KLK14 about 8 times less than that of trypsin. Of note is that although KLK6 exhibited a lower EC$_{50}$ than that of KLK14, indicating a higher potency for PAR$_2$
activation, the maximum calcium signal generated by KLK14 was much greater than that for KLK6 (Figure 15B), suggesting a complex action of KLK6, eliciting both receptor activation (lower KLK6 concentrations) and inactivation (higher KLK6 concentrations). This hypothesis is in agreement with the minor cleavage of rat PAR2 peptide downstream of the tethered ligand (Figure 13B, tracing D, first peak on the right).
Figure 15: (A) Mobilization of intracellular calcium by KLKs, trypsin, and SLIGRL-NH₂ in KNRKrPAR₂ and KNRKrPAR₂(R³⁶A) cells. A. Representative calcium response data triggered by trypsin (Trp, 1U/ml, □), KLK14 (●), KLK6 (▲), KLK5 (■), and Calcium ionophore (CI, I). B. Desensitization of KLK14 action by pre-activation of PAR₂ with SLIGRL-NH₂. C. Activation of calcium signalling by SLIGRL-NH₂ but not by trypsin and KLK14 in KNRKrPAR₂(R³⁶A) cells. The cellular response to calcium ionophore (CI, 2 µM) is shown at the start of tracing A. An upward deflection (arrow on ordinate) indicates an increase in intracellular calcium. The scale for time and calcium signalling in arbitrary units (upward deflection in centimeters) is shown by the inset. On average the basal calcium concentration was 30 nM and the ionophore-triggered peak calcium concentration was in the range of 340 nM. Data are representative of three or more replicate experiments done with independently grown cell cultures. (B) Concentration-effect curves for mobilizing intracellular calcium in KNRKrPAR₂ cells by KLK14, KLK6, and trypsin. The calcium signal, relative to that caused by 2 µM ionophore (% A₂₃₁₈₇), was monitored for increasing enzyme concentrations. Each data point represents the average ± s.e.m. (bars) for three or more measurements at each enzyme concentration, done with separately grown cell cultures. Error bars smaller than the size of the symbols are not shown.
We next examined the ability of KLKs 5, 6, and 14 to activate calcium signalling via human PARs in the HEK-293 cell line (Figure 16, A and B). In contrast with the KNRK-rPAR2 cell line that expresses a high abundance of only one of the PARs, the HEK-293 cells constitutively express both PAR1 and PAR2 (but not PAR4) at levels one might expect in vivo, so the relative selectivity of the kallikrein-related peptidases to activate either PAR1 or PAR2 (or both) could be evaluated. All three KLKs caused a comparable elevation of intracellular calcium in the HEK-293 cells, presumably from the combined activation of PAR1 and PAR2 (Figure 16A, tracing A).

The ability of KLK14 to activate either PAR1 or PAR2 individually was assessed using a receptor desensitization assay (Figure 16A, tracings B–D). In the desensitization assay, the calcium signal caused by KLK14 in the HEK-293 cells after first desensitizing PAR1 with a high concentration (100 µM) of the selective PAR1-activating peptide, TFLLR-NH₂, indicated an activation of PAR2 (Figure 16A, tracing B). Conversely, the calcium signal generated by KLK14 in HEK-293 cells, first desensitized by a saturating concentration of the selective PAR2-activating peptide, SLIGRL-NH₂ (100 µM), demonstrated an activation of PAR1 (Figure 16A, tracing C). Finally, first pre-desensitizing the HEK cells with a high concentration (200 µM) of the non-selective PAR-activating peptide, SFLLR-NH₂, which effects both PAR1 and PAR2, eliminated the KLK14-induced signal entirely, demonstrating that the KLK14-triggered HEK calcium signals did not arise from the activation of receptors other than PAR1 and PAR2 (Figure 16A, tracing D). From the peak heights of the calcium signals observed after pre-desensitization of either PAR1 (PAR2 signal seen in Figure 16A, tracing B) or PAR2 (a much smaller PAR1 signal seen in Figure 16A, tracing C), relative to the integrated signals caused in the cells prior to desensitization (i.e. generated by simultaneous activation of both PARs; see right-hand tracings in Figure 16A, results B and C), it was possible to deduce that the integrated signal
caused by KLK14 acting on both PAR₁ and PAR₂ in the non-desensitized cells came largely from PAR₂ activation. With this same approach, by first desensitizing PAR₁ with TFLLR-NH₂, it was possible to determine the concentration-effect curve for the selective activation of human PAR₂ by KLK14 (EC₅₀ ≈ 8 units/ml; middle curve in Figure 16B); and by pre-desensitizing with SLIGRL-NH₂, the concentration-effect curve for KLK14 activation of human PAR₁ was determined (EC₅₀ ≈ 10 units/ml; bottom curve in Figure 16B). As shown in Figure 16B, the potency of KLK14 for activating a calcium signal via PAR₂ (middle curve in Figure 16B) was close to its potency for signalling via PAR₁ (lower curve in Figure 16B), given the equivalent EC₅₀ values of 8-10 Units/ml. However, the maximum calcium signal caused by the selective activation of PAR₂ by KLK14 at 20 Units/ml was equivalent to the maximum signal caused by the selective activation of PAR₂ by a maximally active concentration (100 µM) of the PAR-activating peptide, SLIGRL-NH₂ (compare the calcium signals shown in the left-hand results of Figure 16A, tracings A and C). In contrast, the maximum calcium signal generated by the selective activation of PAR₁ by KLK14 at its optimal concentration for causing a calcium signal (20 Units/ml) was substantially lower than the maximum calcium signal generated by selectively activating PAR₁ with thrombin (top curve in Figure 16B). The considerably lower maximum PAR₁-derived calcium signal caused by KLK14 relative to that caused by thrombin (or relative to the TFLLR-NH₂ peptide-stimulated calcium signal, compare the left-hand portion of tracing B in Figure 16A with the KLK14-generated signal in tracing C of Figure 16A) suggested a combined activation/dis-arming of PAR₁ by KLK14 (see below). Thus, KLK5, 6, and 14 activate calcium signalling in the HEK-293 cells, with KLK14 causing a strong signal via PAR₂ at concentrations about 8-fold higher than those at which trypsin activates PAR₂. Also, like trypsin, KLK14 signals predominantly via PAR₂ in the HEK-293 cells, causing only a small signal (relative to that triggered by thrombin or TFLLR-NH₂) via PAR₁ activation.
Figure 16: KLKs 5, 6, and 14 signalling in HEK-293 cells; (A) Mobilization of intracellular calcium: reduction in signalling by prior desensitization of PARs 1 and 2. An upward deflection (arrow on ordinate) indicates an increase in intracellular calcium. Panel A. Tracings show representative combined PAR1/PAR2-mediated increases in HEK cell intracellular calcium caused by KLKs 14, 6, and 5. Panel B. PAR2-mediated signalling by KLK14 in HEK cells pre-treated with TFLLR-NH2 to desensitize HEK PAR1. Panel C. PAR1-mediated signalling by KLK14 in HEK cells pre-treated with SLIGRL-NH2 to desensitize PAR2. Panel D. Lack of KLK14 signalling in HEK cells pre-treated with SFLLR-NH2 to co-desensitize both PARs 1 and 2. Tracings are representative of three or more independently conducted experiments done with cells derived from independently grown cultures. The scale for time (min) and calcium signalling (upward deflection in cm) is shown by the inset. (B) Concentration-effect curves for KLK14-mediated mobilization of intracellular calcium in HEK cells by selectively activating either PAR1 (○) or PAR2 (●): comparison with thrombin-mediated activation of PAR1 (⊗). To monitor KLK14 signalling selectively either via PAR1 or via PAR2, HEK cells were first desensitized by either the selective PAR2 agonist, SLIGRL-NH2 (revealing signalling by PAR1: ○, bottom curve) or by the selective PAR1 agonist, TFLLR-NH2 (revealing activation by PAR2 (●, middle curve). Calcium signalling by increasing concentrations of KLK14 in the pre-desensitized cells was monitored. The signalling of KLK14 via PAR1 (bottom curve) can be compared with signalling by thrombin via PAR1 (⊗, top curve). In the HEK cells, thrombin can signal only via PAR1, since it is unable to activate PAR2. Data points represent the average values (± s.e.m.: bars) at each enzyme concentration, obtained for 3 or more measurements with independently grown cell cultures. Error bars smaller in magnitude than the symbols are not shown.
3.3.5 KLK-mediated dis-arming/inhibition of HEK-293 cell PAR₁

Proteolytic removal of the tethered ligand can dis-arm a PAR for activation by its selective proteinase. The much smaller maximal calcium signal generated by KLK14 via selective PAR₁ activation, relative to the maximum PAR₁ signal caused by either thrombin or the PAR₁-activating peptide, TFLLR-NH₂, pointed to a dis-arming/inhibition of PAR₁ by KLK14. We thus tested the ability of pre-treatment of HEK-293 cells with KLK14 to reduce the subsequent calcium signal caused by thrombin activation of PAR₁ in HEK cells. As shown in Figure 17A, pre-treatment of HEK-293 cells with KLK14 at a concentration that did not by itself desensitize the calcium signal caused by TFLLR-NH₂ (i.e. no desensitization of PAR₁; Figure 17A, tracing B, and lower panel, right-hand bar), reduced the subsequent signal caused by thrombin activation of PAR₁ (Figure 17A, tracing A, and lower panel, left-hand bar). It is important to note in interpreting this experiment using the HEK-293 cells expressing both PAR₁ and PAR₂, that unlike trypsin or KLK14, thrombin is not able to activate a calcium signal in the HEK-293 cells via PAR₂. The concentration-effect curve for the ability of KLK14 to dis-arm the ability of thrombin to activate HEK cell PAR₁ is shown in Figure 17B. At a concentration of 5 Units/ml, KLK14 was able to reduce the thrombin-mediated signal (i.e. dis-arming/inhibition) by more than 50%, without desensitizing the cell response to TFLLR-NH₂. If at this concentration of KLK14 the cells were first exposed to the enzyme for 10 min at room temperature and the KLK14 was then removed by washing the cells by centrifugation and resuspension prior to the calcium flux measurement, the thrombin response was also markedly diminished, but the response to TFLLR-NH₂ was retained (not shown). This result indicated a dis-arming of PAR₁, rather than an inhibition of thrombin action either by thrombin proteolysis or by a tight non-catalytic binding of KLK14 to the cleavage activation site that would prevent thrombin access.
**Figure 17:** Dis-arming of PAR₁ by KLK14; (A) Dis-arming by KLK14 of thrombin (tracing A) but not TFLLR-NH₂ (tracing B) activation of PAR₁ in HEK cells. Upper: Cell suspensions in which calcium signalling was monitored were first exposed to a sub-maximally effective concentration of KLK14, followed by the addition of a test concentration of either thrombin (tracing A) or the selective PAR₁-activating peptide, TFLLR-NH₂ (tracing B). The responses of thrombin and TFLLR-NH₂ in the KLK14 pre-treated cells were compared with the ‘control’ responses in untreated cells (right-hand portions of tracings A and B). Lower: The histograms, representing the averaged data from experiments like those depicted in tracings A and B (± s.e.m., bars: n = 3), show the reduction in thrombin (open histogram), but not TFLLR-NH₂ (solid histogram) mediated calcium signalling in cells that were pre-treated with KLK14. The calcium signalling responses after KLK14 treatment were expressed as a percentage (% control) of the signal observed in the same cell suspension that had not been pre-treated with KLK14. Data represent the average of three experiments with independently grown HEK cell cultures. (B) Dis-arming by KLK14 of thrombin activation of PAR₁ (⊕) and KLK14-mediated desensitization of TFLLR-NH₂ PAR₁ signalling in HEK cells: concentration-effect curves for KLK14. HEK cell suspensions prepared for calcium signalling were first exposed at 24 °C to increasing concentrations of KLK14, and the calcium signal was allowed to return to baseline, with re-filling of the intracellular calcium stores (10 min). Test concentrations (EC₅₀) of either thrombin (0.5 U/ml: ⊕, middle curve) or TFLLR-NH₂ (5 μM: ▼, top curve) were then added and the residual PAR₁-mediated calcium signal (⊕, ▼) was monitored as a percentage (% control) of the calcium signal generated in identical cell suspensions that had not been pre-treated with KLK14. For comparison, the concentration-effect curve for PAR₁-mediated signalling by KLK14 alone (⇓) in cells that were first PAR₂ pre-desensitized with SLIGRL-NH₂ is shown in the bottom curve.
The EC\textsubscript{50} value for the ability of KLK14 to dis-arm/inhibit PAR\textsubscript{1} (about 4.5 Units/ml, \textit{left-hand upper curve} in Figure 17B) was lower than its EC\textsubscript{50} value (about 10 Units/ml, \textit{right-hand, upper curve} in Figure 17B) for causing a desensitization of the selective activation of PAR\textsubscript{1} in the same cells by TFLLR-NH\textsubscript{2}. Thus, in cells co-expressing PAR\textsubscript{1} and PAR\textsubscript{2}, KLK14, and presumably the other KLKs, can have complex actions, activating PAR\textsubscript{2} and, depending on the concentration, either dis-arming/inhibiting or activating PAR\textsubscript{1}.

\subsection*{3.3.6 PAR\textsubscript{4}-mediated rat platelet aggregation}

Because rat platelets possess PAR\textsubscript{4}, but not PAR\textsubscript{1} or PAR\textsubscript{2}, thrombin-mediated aggregation can be attributed to PAR\textsubscript{4} activation and not to the activation of other PARs (Hollenberg \textit{et al.}, 2004). We therefore used a rat platelet aggregation assay to evaluate the ability of kallikrein-related peptidases to activate rat PAR\textsubscript{4}. Like thrombin and trypsin that act \textit{via} PAR\textsubscript{4} in this preparation, KLK14 caused aggregation. Surprisingly, however, neither KLK5 nor KLK6 were able to do so at concentrations that could activate PAR\textsubscript{2} (20 Units/ml: Figure 18). The concentration-effect curve for KLK14-mediated rat platelet aggregation (EC\textsubscript{50} \approx 14 Units/ml) showed that its potency was about 2-fold lower than that of trypsin (EC\textsubscript{50} \approx 7 Units/ml) for activating rat platelet PAR\textsubscript{4} (Figure 18, \textit{left-hand curve}).
Figure 18: Aggregation of rat platelets by thrombin, trypsin, and KLK14, but not by KLKs 5 and 6: concentration-effect curves for KLK14. Rat platelet suspensions were exposed to the indicated concentrations of enzymes and aggregation was monitored as an increase (Δ) in light transmission (% maximum change relative to maximal light transmitted in the absence of platelets) as described in Materials and Methods. Aggregation of rat platelet suspensions exposed to increasing concentrations of either trypsin (□) or KLK14 (○) was monitored and expressed as a percentage (% thrombin) of the maximal aggregation caused in the same platelet suspensions by 1 Unit/ml thrombin. Neither KLK6 nor KLK5 caused aggregation at concentrations equivalent to KLK14. Data at each enzyme concentration represent the averages of eight or more aggregation measurements (± s.e.m., bars) done with separately harvested platelet suspensions.
3.3.7 KLK-mediated activation of rat PAR$_4$ expressed in HEK-293 cells

To assess further the ability of KLK14 to activate rat PAR$_4$, we used HEK-293 cells expressing recombinant rat PAR$_4$ in addition to the constitutive expression of human PAR$_1$ and PAR$_2$. Activation of PAR$_4$ with the PAR$_4$-selective activating peptide, AYPGKF-NH$_2$ (which cannot activate either PAR$_1$ or PAR$_2$), caused an increase in intracellular calcium (Figure 19A, tracing B) in cells that had already been desensitized with SFLLR-NH$_2$ to eliminate any signalling by either PAR$_1$ or PAR$_2$. In cells that were first desensitized with SFLLR-NH$_2$, which simultaneously silences signalling by PAR$_1$ and PAR$_2$, KLK14 yielded a robust calcium signal (Figure 19A, tracing A). In the same type of assay, cells pre-desensitized by the combined agonists of PAR$_1$, PAR$_2$, and PAR$_4$ (SFLLR-NH$_2$ plus AYPGKF-NH$_2$) no longer yielded a calcium signal in response to KLK14 (Figure 19A, tracing B, right hand portion). Furthermore, HEK-293 cells that were not transfected with the rat PAR$_4$ construct, upon being desensitized with SFLLR-NH$_2$ (to silence both PAR$_1$ and PAR$_2$), no longer responded to KLK14 (data not shown and result shown in Figure 16A, tracing D). Thus, unequivocally, KLK14 was able to activate PAR$_4$ in the HEK-rPAR$_4$ cells and did not cause its calcium signal via a non-PAR mechanism.

To evaluate the sensitivity of rat PAR$_4$ to activation by KLK14, HEK-rPAR$_4$ cells were PAR$_1$/PAR$_2$ pre-desensitized by treatment with a relatively high concentration of SFLLR-NH$_2$ and were then exposed to increasing concentrations of KLK14 (Figure 19B, right-hand curve). The potency of KLK14 in this assay (EC$_{50} \approx 15$ Units/ml) (Figure 19B, right-hand curve) was in the same range as that of trypsin (EC$_{50} \approx 9$ Units/ml) (Figure 19B, left-hand curve). These results for KLK14-mediated activation of rat PAR$_4$ expressed in HEK-293 cells were entirely in keeping with the ability of KLK14 to activate PAR$_4$-dependent rat platelet aggregation (see
above) and to release a PAR₄- activating sequence from the synthetic rat PAR₄-derived tethered ligand peptide (Table 12).

Figure 19: KLK14 activation of rat PAR₄; (A) KLK14 activation of rat PAR₄ calcium signalling in HEK-rPAR₄ cells. Representative tracings show: Panel A. KLK14-triggered mobilization of intracellular calcium via PAR₄ in HEK-rPAR₄ cells PAR₁/PAR₂ desensitized by pre-treatment with supra-maximal concentrations of the non-selective PAR₁/PAR₂ agonist, SFLLR-NH₂. The KLK14-mediated calcium signal in non-desensitized cells is shown on the right. Panel B. Elimination of KLK14 signalling in HEK-rPAR₄ cells PAR₁/PAR₂/PAR₄ desensitized by pre-treatment with supra-maximal concentrations of the PAR₁/PAR₂ agonist, SFLLR-NH₂, plus the selective PAR₄ agonist, AYPGKF-NH₂. The tracings are representative of three or more independently conducted experiments using separately grown cell cultures. (B) Activation of rat PAR₄ in HEK-rPAR₄ cells by KLK14 and trypsin: concentration-effect curves for mobilization of intracellular calcium. HEK-rPAR₄ cells prepared for calcium signalling experiments were first PAR₁/PAR₂ desensitized with a supra-maximal concentration of SFLLR-NH₂ and the calcium signals generated by increasing concentrations of either trypsin (Trp: □) or KLK14 (○) were then monitored and expressed as a percentage (% A23187) of the calcium signal caused by 2 µM of the ionophore, A23187 in the same cell suspension. Data points at the indicated enzyme concentrations represent the average values (± s.e.m., bars) obtained from four or more replicate cell suspensions derived from independently grown cell cultures. Error bars smaller in magnitude than the symbols are not shown.
To further assess the ability of KLK14 to activate human PAR4, we used a human platelet calcium signalling assay (Covic et al., 2002b). As opposed to an aggregation assay, the calcium signalling assay permits a sequential and concurrent evaluation of the activation of platelet PAR1 and PAR4 in the same platelet aliquot. In the human platelet, which does not possess PAR2, aggregation is triggered by the combined action of PAR1 and PAR4 (Coughlin, 2005). That said, activation of PAR1 and PAR4 can have distinct effects on platelet function (Ma et al., 2005); therefore, we wished to determine whether KLK14 might differentially activate PAR1 and PAR4 in the human platelet preparation. The receptor-selective activating peptides for PAR1 (TFLLR-NH2, Figure 20, tracing A) and PAR4 (AYPGKF-NH2, Figure 20, tracing C) each generated a strong calcium signal in the human platelet preparation, as did KLK14 (20 Units/ml, right-hand portions of results in Figure 20, tracings A–C). When the human platelets were first treated with the PAR1 antagonist SCH 79797, to eliminate either TFLLR-NH2 or thrombin signalling via PAR1 (Figure 20, left portion of tracing B, and data not shown for thrombin), or with a PAR1-desensitizing concentration of TFLLR-NH2 (Figure 20, tracing A), KLK14 (20 Units/ml) still caused a calcium signalling response that was almost the same as that observed in non-desensitized cells (Figure 20, tracings A and B: compare the responses on the far right with the responses to KLK14 in antagonist-treated or PAR1-desensitized cells in the left-hand tracings in each figure). This result indicated that in the human platelet preparation, the main calcium signal generated by KLK14 was triggered by PAR4. In keeping with this result, treatment of the platelets with a PAR4-desensitizing concentration of AYPGKF-NH2 substantially reduced the KLK14-triggered signal, revealing only a very small calcium response (Figure 20, tracing C). As already alluded to, this small residual response in the presence of AYPGKF-NH2 [on average (n = 4) 10% of the signal generated in non-desensitized platelets]
was presumably because of PAR\textsubscript{1} activation. Finally, the combined treatment of the platelets with the PAR\textsubscript{1} antagonist SCH 79797 in combination with a desensitizing concentration of AYPGKF-NH\textsubscript{2} eliminated the KLK14-triggered calcium signal, as well as the signal generated by either thrombin or TFLLR-NH\textsubscript{2} (Figure 20, tracing D).

This result indicated that KLK14 was acting via the PARs and not via another receptor. In contrast with the ability of PAR\textsubscript{4} desensitization to reduce the KLK14 signal by about 90% (Figure 20, tracing C), the human platelet signal generated by thrombin after PAR\textsubscript{4} desensitization, because of PAR\textsubscript{1} activation, was reduced by only about 20% (Figure 20, tracing E). Thus, in contrast with the thrombin-triggered signal generated largely by PAR\textsubscript{1} activation, calcium signalling in human platelets driven by KLK14 appeared to result primarily (about 90%) from activation of PAR\textsubscript{4} and not PAR\textsubscript{1} (compare tracings C and E in Figure 20).

In contrast with KLK14, neither KLK5 nor KLK6 (20 Units/ml) caused a calcium signal in the human platelets, indicating a lack of activation of either PAR\textsubscript{1} or PAR\textsubscript{4}. Furthermore, KLK5 or KLK6 did not trigger human platelet aggregation, whereas KLK14 did (not shown). These results paralleled the lack of action of these two kallikrein-related peptidases in the rat platelet preparation (Figure 18). There was therefore a differential action of the three KLKs in terms of activating human (and rat) PAR\textsubscript{4}.
Figure 20: Mobilization of intracellular calcium in human platelets by KLK14 signalling via PAR4 (tracings A, B) and PAR1 (tracing C): comparison with thrombin activation of PAR1. A. and B. Selective signalling of KLK14 via PAR4. Platelet PAR1 signalling was eliminated either by prior desensitization of PAR1 with TFLLR-NH2 (TF, ▼: tracing A) or by blockade of PAR1 with SCH 79797 (SCH, ▶: tracing B). Residual signalling of KLK14 via PAR4 was then monitored. C. Selective signalling by KLK14 via PAR1. Platelet PAR4 signalling was eliminated by prior desensitization with the PAR4-selective agonist, AYPGKF-NH2 (AYP, △), revealing a small residual PAR1 signal triggered by KLK14. D. Lack of signalling by thrombin and KLK14 after concurrent desensitization of PAR4 and PAR1 with AYP and SCH 79797 (PAR1 antagonist). E. Selective signalling of thrombin via PAR1. Platelet PAR4 signalling was eliminated by prior desensitization with the PAR4-selective agonist, AYPGKF-NH2 (AYP, △), revealing substantial residual PAR1 signal triggered by thrombin. The tracings are representative of three or more identical experiments done with independently harvested human platelet preparations.
In summary, the calcium signalling experiments showed that in human platelets, as in the rPAR₄-expressing HEK-293 cells (Figure 19A), KLK14 can preferentially activate PAR₄ rather than PAR₁, which is in contrast dis-armed/inhibited by KLK14. This selectivity of KLK14 for PAR₄ over PAR₁ in the platelet is the opposite of the selectivity observed for thrombin, which preferentially activates human platelet PAR₁, although also able to activate PAR₄ at higher concentrations (Coughlin, 2005).

3.3.9 Aorta ring relaxation assays using rat and mouse vascular tissue

Activation of either PAR₁ or PAR₂ (Muramatsu et al., 1992; Al-Ani et al., 1995) but not PAR₄ (Hollenberg et al., 2004) induces an endothelium-dependent, nitric oxide-mediated relaxation of rat or mouse aorta. To determine whether KLKs could activate PARs in intact tissues, which unlike the cells may contain extracellular proteinase inhibitors in vivo, we examined the actions of KLKs in rat and mouse aorta preparations. KLK5, KLK6, and KLK14 caused a relaxation of endothelium-intact rat aorta tissue that had been pre-constricted with phenylephrine (Figure 21A, tracing A). As with our previous results for trypsin-mediated PAR-triggered vasorelaxation, the relaxant response caused by KLK14 was absent in endothelium-free preparations (not shown) and was abolished by the nitric-oxide synthase inhibitor L-NAME (Figure 21A, tracing B). KLK5 and KLK6 also caused an endothelium-dependent relaxation that was abolished by L-NAME (not shown).

To explore the mechanism of KLK14-induced relaxation further, we evaluated its ability to relax phenylephrine pre-contracted aorta from wild-type mice (Figure 21A, tracing C) and PAR₂-null mice (Figure 21A, tracing D). The concentration-effect curve for this relaxant action of KLK14 in the wild-type mouse aorta preparation was also determined (Figure 21B), to ensure that the PAR-null tissues had been tested at appropriately high concentrations of KLK14.
Figure 21: (A) KLK-mediated relaxation of rat and mouse aorta preparations: lack of effect in tissue from PAR2-null mice. A. and B. Rat aorta relaxation. A. representative tracings for the relaxant actions of KLKs 5, 6, and 14 in rat aorta tissue with an intact endothelium (relaxant response to 10 µM acetylcholine: ○). B. Relaxant effect of KLK14 is blocked by the NO-synthase inhibitor, L-NAME ( ▼ ). C. and D. Mouse aorta relaxation. C. Representative tracing for relaxation caused by KLK14 in an endothelium-intact mouse aorta preparation (relaxant response to 10 µM acetylcholine: ○). D. Lack of relaxation caused by KLK14, with relaxation caused by thrombin and the PAR1-activating peptide, TFLLRN-NH₂ (TF) in aorta tissue from PAR2-null mice. (B) Concentration-effect curves for KLK14-mediated relaxation in wild-type C57Bl mouse aorta, compared with trypsin concentration-effect curve. The relaxant responses triggered by increasing concentrations of either trypsin (Trp: □ ) or KLK14 ( ○ ) in phenylephrine-constricted endothelium-intact C57Bl mouse aorta preparations were monitored and expressed as a percentage (% Ach) of the relaxant action of 10 µM acetylcholine in the same tissue preparation. Values at each enzyme concentration represent the averages (± s.e.m., bars) of four or more independent measurements on tissue preparations obtained from three or more animals.
In the endothelium-intact mouse aorta preparation, KLK14 caused a relaxation response that was equivalent to that of acetylcholine (Figure 21A, tracing C). The potency of KLK14 for causing a relaxant response in the mouse aorta preparation was close to that of trypsin ($EC_{50} \approx 0.5$ Units/ml; Figure 21B). Concentrations of KLK14 toward the plateau of the concentration-effect curve (3 Units/ml) failed to cause relaxation in pre-constricted tissues from the PAR2-null mice, which otherwise relaxed in response to PAR1 activation by TFLLR-NH$_2$ or thrombin (Figure 21A, tracing D). Thus, KLK14 relaxed the mouse aorta by the selective activation of PAR$_2$ and not via PAR$_1$.

3.4 Conclusions

In this chapter, the central hypothesis of this thesis, which is that KLKs can effect cell signalling via interaction with proteinase-activated receptors (PARs), was investigated following a pharmacological approach. This approach recruited PAR-expressing cells and tissues to lead to the following two major conclusions:

A. KLKs can mediate activation of PARs 1, 2, and 4. The main finding of our study was that signalling via human, rat, and mouse PARs can be regulated by human kallikrein-related peptidases; for instance, KLK14 can target the activation of PAR$_2$ and PAR$_4$ and the disarming/inhibition of PAR$_1$ (see below). We established unequivocally the ability of the KLKs to regulate PAR activation through a number of independent biochemical, cell biological, pharmacological, and genetic approaches as follows:

1. HPLC and mass spectral analysis of the fragments yielded upon incubation of KLKs 5, 6, and 14 with PAR N-terminal peptide sequences based on the cleavage/activation motifs of PARs 1, 2, and 4.
2. PAR-dependent calcium signalling responses in cells expressing PARs 1, 2, and 4.

3. A vascular ring vasorelaxation assay using rat and mouse tissue, as well as tissue from PAR2-null mice; and

4. A PAR4-dependent rat and human platelet aggregation assay.

The actions of the KLKs are in marked contrast to the action of plasma kallikrein (a genetically distinct enzyme), as well as tissue kallikrein (KLK1), which are reported not to activate PAR2 (Molino et al., 1997b). Given the conservation between the genetic loci of the KLKs 4-15 in non-human species like the mouse (Olsson and Lundwall, 2002; Elliott et al., 2006; Lundwall and Brattsand, 2008), we suggest that non-human kallikrein-related peptidases that are orthologues of KLKs 5, 6, and 14 would also be able to signal via the PARs with potency comparable with that of pancreatic trypsin. To generalize our findings for a variety of species, we validated the ability of the KLKs to effect PARs of human, rat, and mouse origin, presuming that, just as is the case for human thrombin and trypsin, PARs from other species although not identical in sequence would nonetheless also be susceptible to kallikrein-related peptidase activation. It can be anticipated that, as is the case for signalling via PARs by thrombin and trypsin, the activation by KLKs of calcium signalling via G_q also implies a potential activation of G_i and other downstream signalling events, like the stimulation of mitogen-activated protein kinase, known to be regulated via PAR1 and PAR2.

Our focus was principally on PAR2, known to play an important role in inflammation and nociception (Vergnolle et al., 2001a,b; Vergnolle, 2004), and on KLK14, known to be elevated in patients with ovarian or breast carcinoma (Borgoño et al., 2003; 2007c). In a number of respects, the actions of KLK5 and KLK6 paralleled those of KLK14, particularly in terms of activating PAR2. That PAR2 and its R/S cleavage activation sequence were the targets for KLK action was established as follows:
1. By the lack of cleavage/activation by KLKs of the R^36A mutant of rat PAR_2 in which the tryptic cleavage/activation site was changed to a bond not susceptible to tryptic cleavage (Figure 15A, tracing C).

2. By the lack of a relaxation response of endothelium-intact aorta rings derived from PAR_2-null mice that otherwise relaxed in response to PAR_1 activation (Figure 21A, tracings C and D).

3. By the disappearance of the N-terminal peptide sequence released by tryptic cleavage of rat PAR_2 (reduction of SLAW-A reactivity caused by KLKs), with retention of the amino acid epitopes in the active tethered ligand sequence of rat PAR_2 recognized by the B5 antibody (Figure 14).

The three KLKs yielded comparable results for activation of PAR_2 but demonstrated different potencies. For example, in the assay for PAR_2 activation in the KNRK-rPAR_2 cells (Figure 15B, calcium signalling), KLK6 was less potent than trypsin but more potent than KLK14. As already pointed out, however, the maximum response generated by KLK6 (calcium signal) was lower than that of KLK14. The lower maximum response to KLK6 activation compared with KLK14 is possible to result from a competing increased rate of a dis-arming cleavage at higher KLK6 concentrations.

Human and rat PAR_4 also served as a target for KLK14-mediated activation but not for KLK5 and KLK6. The ability of KLK14 to activate PAR_4 was demonstrated both by the rat platelet aggregation response to KLK14, which is PAR_4-dependent (Hollenberg et al., 2004), and by the calcium signalling responses in rat PAR_4-expressing HEK-293 cells (Figures 18 and 19). The results with the HEK-293 cell calcium signalling assay showed that the potency of KLK14 for activating rat PAR_4 (EC_{50} ≈ 15 units/ml; Figure 19B) was comparable with but lower than its potency for activating either rat or human PAR_2 (EC_{50} ≈ 8 units/ml; Figures 15B
and 16B). In the human platelet calcium signalling assay, KLK14 was also able to activate PAR4, as indicated by the calcium signal generated in the presence of a PAR1 antagonist and from the data obtained using the PAR4 desensitization protocol (Figure 20). As already mentioned, neither KLK5 nor KLK6 was able to activate either rat or human PAR4, indicating differences between the KLKs for targeting the PARs.

The actions of KLK5 and KLK6 were distinct from those of KLK14 in terms of their inability to activate PARs 1 and 4. More specifically KLKs 5 and 6 did not cause any activation signal via PARs 1 and 4 in our cell culture systems and isolated platelets. The lack of activation of PAR4 by these two kallikrein-related peptidases is in keeping with the very minor cleavage by KLK5 and KLK6 we observed by HPLC analysis for the PAR4-derived peptides and the lack of hydrolysis by KLK6 of a model PAR4 peptide described in recent work (Angelo et al., 2006). A possible dis-abling or dis-arming of PAR1 by KLKs 5 and 6 remains a question for future investigation.

**B. KLK14 causes preferential dis-arming/inhibition of PAR1.** The HPLC analysis of the KLK14 cleavage products of hPAR1 demonstrated a much lower degree of cleavage than for PAR2 and PAR4. Notwithstanding, KLK14 hydrolysis of the PAR1 peptide yielded about equal amounts of peptides resulting from cleavage at sites that would either activate or dis-arm the receptor (Figure 13A), whilst a similar dis-arming peptide was not generated by KLK5 or KLK6. In the HEK-293 cells and human platelet test systems, it was clear that KLK14 preferentially dis-armed/inhibited PAR1 rather than causing activation. This result paralleled exactly our previous data obtained for the dis-arming of PAR1 by trypsin (Kawabata et al., 1999). The data demonstrating that the KLK14-treated HEK-293 cells, when washed free from the enzyme, were no longer sensitive to thrombin but responded well to the PAR1-activating peptide, TFLLR-NH2, pointed to removal of the PAR1 tethered ligand, as predicted by the
peptide proteolysis data. This preferential dis-arming/inhibition of PAR\textsubscript{1} by KLK14 is similar to the ability of neutrophil elastase to dis-arm/inhibit PAR\textsubscript{2} (Dulon et al., 2003).

Our LC-MS and MALDI analysis of the complex PAR peptide proteolysis mixture generated by the KLKs revealed unexpected non-tryptic cleavage products that we propose are only minor constituents, because we did not detect the peptides by HPLC analysis. These minor cleavage sites could also dis-arm the PARs. For instance, the cleavage of the hPAR\textsubscript{1} peptide at the SF/LL bond by KLK14 that would dis-arm PAR\textsubscript{1} may reflect the KLK14-suggested minor chymotryptic activity (Felber et al., 2005). Whether these minor cleavages would play a role in regulating PAR activity \textit{in vivo} remains an open question.

In summary, we have concluded that the kallikrein-related peptidases can target PARs and exhibit different potencies and distinct actions. More specifically, they can cause:

1. Strong activation of PAR\textsubscript{2} (KLKs 5, 6, and 14)
2. Dis-arming and activation of PAR\textsubscript{1} (KLK14), depending on the local concentration and relative rates of cleavage at each site
3. Signalling via PAR\textsubscript{4}, both in platelets (rat, human) and in PAR\textsubscript{4}-expressing cells (KLK14).

It should be emphasized that even though the members of the KLK family are highly conserved structurally (30 to 50\%), with similar trypsin or chymotrypsin-like specificity and common substrates and inhibitors, their enzymatic functions \textit{in vivo} can be essentially diverse. This in part can be attributed to the variable external surface loops surrounding the substrate-binding site, which may control their activity, define substrate and inhibitor specificity, and function in autolytic regulation, or to the presence of possible co-factors, like zinc, which have the ability to effect proteinase activity (Borgoño et al., 2004; Borgoño and Diamandis, 2004).

The data derived from our pharmacological study suggested that distinct KLKs not only target different members of the proteinase-activated receptor family, but also exhibit variable
pharmacological potencies against the same site of catalysis. As a proof of principle our recent unpublished data indicate that KLKs 1 and 12 interact differently with PARs; for example, KLK12 seemed to target preferentially PAR$_2$ at a site that causes inhibition of receptor activation by trypsin. We therefore anticipate that the other human kallikrein-related peptidases, apart from KLK5, KLK6, and KLK14, as well as KLKs from other species will have both common and distinct actions via the different PARs in cells and tissues expressing these receptors. Taking this PAR-KLK interaction into account, the next important steps were to investigate if KLKs can act in vivo and even further if they are active in vivo.
Chapter IV.

Confirm PARs as KLK Cell Surface Targets *In Vivo*

ORIGINAL PUBLICATIONS:
CONFIRM PARs AS KLK CELL SURFACE TARGETS IN VIVO

4.1 Introduction

Although the pharmacological studies done in vitro may indicate what can potentially happen in a physiological setting, it may be misleading to establish the role of proteinases, like the KLKs, on regulation of PARs based solely on these data. More specifically, in the previous section (Chapter III) in vitro synthetic peptides, corresponding to the PAR activation site, intact cells (cell culture lines and isolated human and rat platelets) and tissues, over-expressing the PARs, were utilized to prove that kallikrein-related peptidases can initiate signalling via these receptors. However, all of the above protocols include an unavoidable bias, since they are targeting one or two receptors, which are over-expressed in these systems. Furthermore, all of the above cell systems were utilized in suspension, independently of the matrix network which can release components capable of regulating cell behaviour. In addition, cell lines, platelets, and tissues were assayed after extensive washing, which decreases the chances of interaction with serine proteinase inhibitors. It was therefore imperative to challenge our hypothesis in a natural system which, apart from PARs, expresses various cell surface receptors, as well as other proteolytic targets. Our additional challenge was to include in our system the potential regulatory role of serine proteinase inhibitors.

We chose to work with a breast cancer cell line, MDA-MB-231, which expresses PARs 1, 2, and 4 (Kamath et al., 2001). These cells have served as a well-established model of studying the role of PARs on the invasive properties of breast tumour cells (Morris et al., 2006). We anticipated that these cells would also express several other receptors related to carcinogenesis; a brief screening of the literature identified two major players, the EGF (Hirsch
et al., 2006) and VEGF (Timoshenko et al., 2007) receptor. Our aim was to identify the cell surface receptors, amongst the proteolytic targets of KLKs in the MDA-MB-231 breast cancer-derived human cell line.

We also used a paw oedema protocol to study the response to treatment by kallikrein-related peptidases in a mouse model of inflammation. From previous work we were confident that on this system, trypsin can cause an inflammatory response via PAR2 (Vergnolle et al., 1999b, 2001b; Cenac et al., 2002, Nguyen et al., 2003). Based on our comparative to trypsin function of KLKs on rodent and human cell systems expressing PAR2, we anticipated that KLKs could also mimic the action of trypsin against PAR2 in the murine model of inflammation.

4.2 Materials and Methods

4.2.1 Proteomic identification of cell surface PAR-derived fragments from tumour cells

MDA-MB-231 (HTB-26; ATCC, Rockville, MD, USA) cells were grown in 75-cm² flasks to 80% confluency using RPMI medium supplemented with 10% fetal bovine serum. Trypsin was not used for sub-culturing the MDA-MB-231 cells, since this could possibly effect the integrity and absolute numbers of the cell surface receptors. Instead, a non-enzymatic detachment of cells was performed using a 10-min treatment of 4 ml of Hank’s dissociation solution (Invitrogen). Mechanical force was additionally used to expedite cell detachment. Cells were subsequently grown until they reached 80% confluency. They were then washed with phosphate buffered saline and incubated with serum-free medium (CDCHO supplemented with 8 mM L-Glutamine; Invitrogen), followed by treatment with 1 µg/ml KLK14 (about 18 Units/ml; Units of trypsin-like activity as defined in section 3.2.2), as a representative
kallikrein-related peptidase, or trypsin for comparison purposes. Treatment with vehicle was also included as a control.

Supernatants (20 ml) were collected after 2 h and were clarified by centrifugation. Samples were lyophilized and resuspended in 8 M urea (322 µl; Sigma) for protein denaturation. We subsequently added 50 µl of one part DTT (200 mM; Sigma) and one part NH₄HCO₃ (1 M), to facilitate the dissolution of the disulfide bonds, and reduced at 50°C for 30 min. Finally, we alkylated with 125 µl of 500 mM iodoacetamide (Sigma) for 1 h at room temperature (on a shaking rotor).

Processing of the samples was carried forward by desalting through a NAP5 column (final volume of 1 ml; GE Healthcare Bio-Sciences Corp.) according to manufacturer’s instructions. In brief, the NAP5 column was first conditioned with 10 mL of 50 mM NH₄HCO₃, and 500 µl of the sample was loaded onto the column, followed by the elution of the proteins using 1 mL of 50 mM NH₄HCO₃. Samples were lyophilized to dryness and enzymatically digested overnight at 37°C, using 50-fold less trypsin (Promega, Madison, WI, USA) than the total protein content of the samples (approx. 1 mg) and additional reagents as indicated below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
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<tbody>
<tr>
<td>50 mM NH₄HCO₃</td>
<td>250 µl</td>
</tr>
<tr>
<td>100% Methanol</td>
<td>100 µl</td>
</tr>
<tr>
<td>ddH₂O water</td>
<td>150 µl</td>
</tr>
<tr>
<td>Trypsin (stock of 1 µg / µl)</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Samples were again lyophilized for 2-3 h and fractionated through a strong cation exchange HPLC column (PolySULFOETHYL A, 200-Å pore size and 5-µm diameter; The Nest Group Inc., Southborough, MA, USA) as per the instructions of the manufacturer. In brief, the dry samples were resuspended in 120 µl of 0.26 M formic acid in 10% acetonitrile (running buffer). The samples were loaded onto the HPLC column. A 1-h fractionation procedure with a
two-step linear gradient of running buffer and 1 M ammonium formate added as the elution buffer was used. Elution was monitored at a wavelength of 280 nm. Samples were collected in glass vials, clarified by passage through C_{18} extraction (ZipTip_{C18} pipette tip, Millipore; catalogue number ZTC18S096) and eluted in 4 µl of 68% acetonitrile (ACN) made up of about 3 parts of Buffer A (95% water, 0.1% formic acid, 5% ACN, 0.02% TFA) and 7 parts of Buffer B (90% ACN, 0.1% formic acid, 10% water, 0.02% TFA). Finally, 80 µl of Buffer A were added to dilute the samples and the peptide/protein content was analyzed by liquid chromatography / tandem mass spectrometry (LC-MS/MS; LTQ Thermo-Finnigan; Thermo Fisher Scientific Inc., Waltham, MA, USA), as previously reported (Kulasingam and Diamandis, 2007). Raw data were assigned to proteins included within the human protein database Swiss-Prot using the Mascot algorithm (Matrix Science Inc., Boston, MA, USA) and probabilities were further evaluated with Scaffold (Proteome Software Inc., Portland, OR, USA). Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 80% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003).

4.2.2 Mouse model of inflammation and pain

Under the same methodology previously employed to assess the inflammatory action of agonists of PAR_{4} (Hollenberg et al., 2004; Houle et al., 2005), we used the murine paw oedema model to examine the potential inflammatory action of kallikrein-related peptidases via PARs. C57bl/6 mice (4-6 weeks old; 20-25 g) were obtained from Charles River Laboratories (Montreal, QC, Canada) and the PAR_{3}-null animals were a kind gift of Johnson and Johnson
The rodents were bred under the same conditions for more than 10 generations, had free access to food and water and were housed under constant temperature (22°C) and photoperiod (12 h light–dark cycle). All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

For this study we elected KLK14 as a representative KLK to study their inflammatory properties in this system, mainly due to the enzyme’s high capability of targeting PARs 1, 2, and 4. Prior to the administration of the KLK14, a basal measurement of the paw thickness of each mouse was recorded using an electronic calliper (Fisher Scientific, Hampton, NH, USA). Control mice littermates were treated with heat-inactivated KLK14 (boiling for 10 min) or buffer alone (n=5 mice per group). Each compound was administered via intraplantar injection into the paw, diluted in sterile saline (0.9% NaCl) in a final volume of 10 µl per mouse paw (for KLK14: 18 Units per paw). As an index of oedema formation, paw thickness was then measured every 15 min for up to 1 h after the injection. Oedema was followed for a total of 4 h (hourly measurements were performed after the first hour).

The effect of KLK14 on nociception was also evaluated using the same rodent model, as previously described (Asfaha et al., 2007). Each mouse was placed individually in a clear plastic testing box and was allowed to acclimatize to the new environment for a minimum of 10 min. The age and sex-matched littermates were treated with active (right paw) or control heat-inactivated (left paw) KLK14 by intraplantar injection (n=4 per group) as above. Responses to a thermal stimulus were evaluated by measuring paw withdrawal latency in response to a radiant heat stimulus applied using a ‘plantar test’ apparatus (Ugo Basile, Milan, Italy). Nociceptive responses to a mechanical stimulus were assessed using von Frey monofilaments with bending
forces of 0.4, 0.6, and 1 g, measuring mechanical nociceptive flexion reflex. The filaments were
applied three times randomly among the tested mice for 1 to 2 sec before (time=0) and after the
injections. A score was assigned based on the animal’s response: 0=no movement, 1=paw
movement, and 2=removal of the paw accompanied with paw licking or vocalization.
Mechanical nociceptive score was expressed as the sum of the score for the three applications
(score ranging from 0 to a maximum of 6). Before any experiments, baseline measures (time=0)
were recorded. The paw withdrawal latency and nociceptive threshold were then evaluated at
30-min intervals for 1 h after the injection. Measurements were also considered hourly for
additional 5 h. For these assays, thermal and mechanical hyperalgesia were defined as a
significant decrease in withdrawal latency and nociceptive threshold or increase of score,
respectively. The opposite effect, an increase of threshold or decrease of score respectively,
defines analgesia.

4.3 Results

4.3.1 Cell surface targets of KLK14 in PAR-expressing tumour cells

Treatment of MDA-MB-231 cells with KLK14 resulted in the fragmentation of about 70
target molecules (25 of these proteins were unique comparing to the vehicle-treated control).
Proteomic analysis of the fragments led to the identification of 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 or IGFBP-7). Amongst the reservoir of other cell surface
targets, PAR$_2$ was successfully identified (Table 13). More specifically the amino terminal
peptides SLIGKVDGTSHVTGK and VDGTSHVTGK were identified with 95% probability
and a 40.9 to 42.3 mascot identity score. The first one of these peptides contains the PAR$_2$
activation tethered ligand sequence (SLIGKV). The second peptide is the result of proteolytic fragmentation within the tethered ligand (pointing to a potential receptor dis-arming); however, due to the enzymatic digestion of the collected cell supernatants, it is uncertain whether this site was a target of proteolysis for KLK14 or for the trypsin introduced later in the sample.

Table 13: Mass spectral protein identification upon treatment of MDA-MB-231 cells with KLK14. Selected protein hits with estimated probabilities over than 94% are included.

<table>
<thead>
<tr>
<th>Proteins common to KLK-treated and non-treated samples</th>
<th>Proteins unique to KLK-treated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin, cytoplasmic 1</td>
<td>Coagulation factor V</td>
</tr>
<tr>
<td>ALB protein</td>
<td>Complement C4-A precursor</td>
</tr>
<tr>
<td>CD44 antigen isoform 4 precursor</td>
<td>Dynein heavy chain, cytosolic</td>
</tr>
<tr>
<td>CYR61 protein</td>
<td>Galectin-3-binding protein precursor</td>
</tr>
<tr>
<td>Elongation factor 1-alpha</td>
<td>Gelsolin precursor</td>
</tr>
<tr>
<td>Fibrinogen beta chain precursor</td>
<td>Insulin-like growth factor-binding protein 7 precursor</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Interferon-induced protein with tetratricopeptide repeats 1</td>
</tr>
<tr>
<td>Hemoglobin alpha subunit</td>
<td>Proteinase-activated receptor 2 precursor</td>
</tr>
<tr>
<td>Kinesin-like protein 2</td>
<td>Receptor-type tyrosine-protein phosphatase F precursor</td>
</tr>
<tr>
<td>Splice Isoform 1 of Heat shock cognate 71 kDa protein</td>
<td>Serine/threonine-protein kinase PLK1</td>
</tr>
<tr>
<td>Splice Isoform 1 of Tubulin alpha-2 chain</td>
<td>Splice Isoform 1 of Periostin precursor</td>
</tr>
<tr>
<td>Splicing factor, arginine/serine-rich 4</td>
<td>Splice Isoform 2 of Basigin precursor</td>
</tr>
<tr>
<td>Testis-specific serine/threonine-protein kinase 6</td>
<td>Thrombospondin-1 precursor *</td>
</tr>
<tr>
<td>Thrombospondin-1 precursor *</td>
<td>Transcriptional repressor protein YY1</td>
</tr>
</tbody>
</table>

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

Intraplantar administration of kallikrein-related peptidase 14 in wild-type mice resulted in an oedema response (Figure 22). We noticed an increased paw diameter of 0.37 ± 0.12 mm (average increase in diameter ± s.e.m., n=5) at 30 min. The swelling was maintained at 60 min (0.50 ± 0.13 mm). The increase in oedema caused by KLK14 (Figure 22) was in keeping with the oedema caused by trypsin, which is not able to cause oedema in PAR2-null mice, over the same time course (data not shown). Administration of the same amount of heat-inactivated KLK14 resulted in lower levels of inflammatory response at the 30-min (0.024 ± 0.01 mm) and 60-min time points (0.05 ± 0.01 mm). This lack of swelling was in stark contrast ($p<0.005$) to the persistent oedema caused by active KLK14.

![Figure 22. KLK14-induced mouse paw oedema.](image)

The increased paw oedema ($\Delta$, mm) in response to KLK14 administration was measured as outlined in the text at 30 min and 60 min for either active (group 1 - left histograms) or heat-inactivated KLK14 (group 2 - right histograms) (18 Units/paw). The bars in the histograms represent the average increases ($\Delta$, mm ± s.e.m., n=5 for group 1 and group 2) in paw diameter.
Nevertheless, in PAR2-null animals KLK14 administration resulted in an increased oedema response (not shown), along with a decreased threshold of thermal nociception, which to a lesser extent was also observed in wild-type mice (Figure 23). The data derived from the nociception measurements after mechanical stimulation were inconclusive.

Figure 23. KLK14-induced hyperalgesia in wild-type and PAR2-null mice. Withdrawal latency, as an indication of increased nociception in response to KLK14 administration, was measured at 30 to 60-min intervals for either active or heat-inactivated KLK14 (18 Units/paw). The bars in the histograms represent the average increases ($\Delta$, mm ± s.e.m., n=4 for all groups) in withdrawal latency. (▲), active KLK14-wild type animal; (■), heat-inactivated KLK14-wild type animal; (△), active KLK14-PAR2-null animal; (□), heat-inactivated KLK14-PAR2-null animal.

4.4 Conclusions

This part of the study confirmed KLKs as PAR regulators in systems, where there might be abundance of cell receptors, serine proteinases, and their respective proteinase inhibitors. We specifically wanted to examine whether kallikrein-related peptidase 14 would be able to
preferentially select one or more of the PARs amongst a plethora of proteolytic targets. We chose to use a concentration of KLK14, which would achieve a close-to-maximum activation of PAR₂, according to the concentration effect curve presented in section 3.3.4, so that we could ensure sufficient amounts of the cleaved peptide in the cell culture supernatant. At this concentration PARs 1 and 4 could also be a target for activation. The incubation of the MDA-MB-231 cells with KLK14 confirmed biochemically the ability of KLK14 to preferentially target PAR₂, amongst the other PAR and non-PAR-related substrates expressed by these cells (Table 13).

In the mouse model of paw inflammation kallikrein-related peptidase 14 was able to overcome the presence of serine proteinase inhibitors and cause significant paw oedema. The pattern of oedema response over time was the same as that previously shown for PAR-induced oedema (Vergnolle et al., 1999b; Hollenberg et al., 2004). We concluded that, as for trypsin and tryptase, KLK14 can cause an inflammatory response in murine paw. As suggested by our in vitro data showing PAR activation by KLK14 and the large volume of data in the literature demonstrating a pro-inflammatory role for PAR activation (Vergnolle, 2001b; Steinhoff et al., 2005; Vergnolle, 2005), we speculated that this pro-inflammatory effect of KLK14 could be mediated by PAR activation. Since, however, a large body of literature also suggests that different proteinase-activated receptors can have either pro- or anti-inflammatory roles in several settings responding to a different proteinase, it is imperative in the future that the action of KLKs in the murine model of inflammation is investigated thoroughly with the inclusion of the appropriate PAR-null animals. Furthermore, our preliminary data of increased inflammatory and pain response in the PAR₂-null mice led to conclusions that KLK14 may target other cell surface receptors (non-PARs or other PARs apart from PAR₂) or other mediators to induce
inflammation in this murine model, which stresses the importance of studying the role of KLK14 in PAR1-null and PAR4-null animals.

In general, the experiments of Chapter III together with those at Chapter IV confirmed the potential of kallikrein-related peptidases to signal via proteinase-activated receptors in vivo. This finding is of particular interest because of the wide distribution of KLKs in many human tissues, the up-regulation by sex steroid hormones, and the increase in a variety of pathophysiological conditions (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Shaw and Diamandis, 2007). Thus, KLKs may represent endogenous PAR modulators in a variety of settings, including cancer, where other trypsin-like enzymes are scarce. The next question therefore to ask is whether enzymatically active KLKs, are present in vivo at concentrations sufficient to regulate tissue function via the PARs.
Chapter V.
Quantification of Active KLKs in Biological Fluids

ORIGINAL PUBLICATION:
QUANTIFICATION OF ACTIVE KLKS IN BIOLOGICAL FLUIDS

5.1 Introduction

Given the potential impact that an active KLK enzyme may have via the PARs, a key issue to consider is: what proportion of the immunoreactive KLK represents active enzyme in human samples? Or in other words what are the physiological concentrations of active KLKs? To investigate this question, we elected KLK6 as a prime candidate, due to its abundant expression in many tissues and its relation to diseases ranging from Alzheimer’s disease to ovarian cancer (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Shaw and Diamandis 2007).

It is well-appreciated that KLK activity is finely regulated, mainly through proteolytic processes of activation and inactivation, as well as by binding to inhibitors (Borgoño and Diamandis, 2004; Borgoño et al., 2004; Luo and Jiang, 2006). For instance, KLK6 is secreted as a zymogen and is activated by the removal of five amino acids from its N-terminal sequence (Gomis-Ruth et al., 2002). KLK6 can auto-catalytically activate via a two-step process (Bayés et al., 2004); however, the rate of this auto-activation is minimal in comparison to the rate of its auto-degradation (Blaber et al., 2007). At least one of these intrinsic cleavage sites of KLK6 that can lead to protein degradation and inactivation has been identified (Berrett et al., 2002; Magklara et al., 2003; Bayés et al., 2004). As with the other KLKs, the activity of KLK6 can be negatively-regulated not only by proteolytic processing of the active enzyme, but also by the inhibitory serine proteinase inhibitors (serpins: Law et al., 2006), such as α2-antiplasmin (Magklara et al., 2003), α1-antichymotrypsin (Hutchinson et al., 2003), and antithrombin III (Magklara et al., 2003).
Although evaluated for the isolated KLK enzymes in vitro (Bernett et al., 2002; Magklara et al., 2003; Bayés et al., 2004; Michael et al., 2005; Blaber et al., 2007, Borgoño et al., 2007c; Yoon et al., 2007; Emami and Diamandis, 2008), the KLK activation and inactivation processes have yet to be monitored in vivo. Furthermore, as mentioned above, in spite of the extensive literature dealing with the abundance of immunoreactive KLKs in a variety of normal and cancer-related settings, there is as yet little information available on the proportion of immunoreactive KLKs that represent active enzymes in such samples. As a result, the specific pathway(s) of activation/de-activation of the KLK family in vivo are not yet clear.

The mechanisms whereby enzymatically active KLKs, including KLKs 5, 6, and 14, may regulate tissue function in vivo are the subject of much interest (Hansen et al., 2008b; Hollenberg et al., 2008). Some effects of KLKs in the context of cancer progression are believed to be due to the cleavage of a number of extracellular targets, such as proteins of the extracellular matrix, pro-urokinase-plasminogen activator, kininogens, growth factor precursors (and binding proteins), and other KLKs (Borgoño and Diamandis, 2004). In addition, our own work revealed that the KLKs, including KLKs 5, 6, and 14, can signal to cells via proteinase-activated receptors (Chapter III). Activation of these G protein-coupled receptors can initiate multiple signalling pathways related to inflammation, carcinogenesis, invasion, metastasis, angiogenesis, cell death and survival (Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005; Hansen et al., 2008b; Ramachandran and Hollenberg, 2008). Thus, we suggest that an understanding of the pathophysiological role of KLKs in vivo will require measurements not only of the total immunoreactive levels of enzyme that may be present, but also of the proportion of enzyme that is present in its catalytically active state, which can regulate tissue signalling by both PAR-dependent and PAR-independent mechanisms.
To monitor the presence of enzymatically active serine proteinases such as the KLKs, in cell- or tissue-derived samples, as those utilized in Chapters II, III, and IV, we elected to use probes of enzyme activity (activity-based probes or ABPs). These probes irreversibly modify the enzyme active-site nucleophile and, by virtue of a reporter tag, allow the detection and purification of individual labelled enzymes through simple separation techniques such as SDS-PAGE (Baruch et al., 2004; Hansen et al., 2005; Pan et al., 2006; Sadaghiani et al., 2007). In Chapter II, with the use of activity-based probes (ABPs), we managed to monitor the conversion of KLK6 from its zymogen to its enzymatically active species and to develop an improved procedure for the isolation of active KLK (free from its zymogen). The later section focuses on KLK6, as a prototype member of the KLK family with serine proteinase activity, to develop an analytical approach using a serine proteinase-targeted ABP coupled to immunoassay measurements. Our aim was to quantify the proportion of enzymatically active KLK6, relative to total immunoreactive KLK6, which is present in ovarian cancer-derived ascites fluid, CSF, and supernatants from cancer-related cell cultures (Okui et al., 2001, Hutchinson et al., 2003, Luo et al. 2006; Shaw and Diamandis, 2007; our unpublished data).

5.2 Materials and Methods

5.2.1 Biological samples

All clinical specimens were collected upon Ethics Approval from the Mount Sinai Hospital Institutional Review Board. Ascites samples were collected with paracentecis from ovarian cancer patients at Princess Margaret Hospital, Toronto, ON, Canada in 500-ml vacuum bottles. Residual cerebrospinal fluid samples (collected by lumbar puncture) in excess of the amounts required for routine testing were obtained at Mount Sinai Hospital, Toronto, ON,
Canada. Two ovarian cancer cell lines; HTB-75 (Caov-3) and HTB-161 (OVCAR-3), as well as one breast cancer cell line; HTB-132 (MDA-MB-468), were obtained from the American Type Culture Collection (ATCC). The androgen receptor-transfected prostate PC-3(AR)₆ cell line was a generous gift from Dr. Theodore Brown (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, and the University of Toronto, Toronto, ON, Canada) (Heisler et al., 1997). All cell lines were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA), supplemented with fetal bovine serum (10%), in 75-cm² flasks. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere until the cell monolayers became confluent and supernatants were collected. All samples were clarified by centrifugation (10 min, 4000 rpm) and stored at -20°C until use.

5.2.2 Combined activity-based probe ELISA assay of KLK6-containing samples

Each sample was assayed independently (a) for total immunoreactive KLK6, measured by our in-house ELISA assay, and (b) for total activity-based probe-reactive biotinylated KLK6 measured by a combined antibody capture / streptavidin-based biotin microtiter plate assay. A standard curve for KLK6 was prepared for these assays using the active KLK6 enzyme prepared by our new two-step purification procedure (section 2.2.5, Protocol B).

KLK6 sandwich-type ELISA assay

The sandwich-type ELISA assay for measuring total amount of KLK6, used a capture antibody that has been previously developed in our laboratory, as described elsewhere in detail for its KLK6 selectivity and lack of cross-reactivity with other kallikrein-related peptidases (Diamandis et al., 2000d; Luo et al., 2006). In the present study, two in-house monoclonal antibodies against KLK6 were utilized (Luo et al., 2006). A time-resolved fluorometer, the
CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada), was used to measure Tb^{3+} fluorescence in white microtiter 96-well plates, as described in detail in section 2.2.2.

**KLK6 activity-based probe ratiometric ELISA assay (KLK6 ABRA-ELISA)**

Several activity-based probes for trypsin-like serine proteinases, which were available in our laboratory, were screened to select for the probe with the highest sensitivity against KLK6. More specifically, we utilized four biotinylated diphenylphosphonate probes containing Pro-Lys, Lys-Lys, Asp-Lys, and Succinyl-Lys as the P₂ and P₁ residues, respectively. The first three probes had similar sensitivity and we therefore decided to continue this study with the Pro-Lys (biotin-linker-Pro-Lys-diphenylphosphonate or Bio-PK) only.

KLK6 calibrators were prepared by diluting recombinant active KLK6 protein produced as described in Chapter II (activity equal to 3500 nmol of VPR-AMC/min/mg of KLK6, which is equivalent to 1 Unit of trypsin-like activity / µg KLK6 when compared to trypsin against VPR-AMC; section 3.2.2) in bovine serum albumin buffer (6% BSA, 50 mM Tris, 0.05% sodium azide, pH 7.8). Where appropriate, sample dilutions were also performed in the 6% BSA buffer. Coating antibody solution (100 µl of 50 mM Tris buffer, 0.05% sodium azide, pH 7.8, containing 500 ng/well antibody) was added to each well and incubated for 8 h at room temperature, as previously described (Diamandis et al., 2000d). Calibrators or samples of unknown active KLK6 content were diluted 2-fold in 5 µM of activity-based probe in KLK6 activity buffer (50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween-20, pH 7.6) and were incubated for 8 h at room temperature prior to addition to the antibody ‘capture’ plate. The plate, coated with a KLK6-selective monoclonal antibody (Luo et al., 2006), was first washed two times with the washing buffer (5 mM Tris buffer, 150 mM NaCl, 0.05% Tween-20, pH 7.8), and the ABP-reacted standards or ‘unknown’ samples were added onto the ‘capture’ plate.
and incubated overnight at room temperature. Subsequently, 5 ng/well of alkaline phosphatase-conjugated streptavidin (SA-ALP) solution (Jackson ImmunoResearch, West Grove, PA, USA) in BSA buffer was added to each well and incubated for 15 min at room temperature. The plate was washed six times with the washing buffer, and the substrate diflunisal phosphate [100 µl of 0.1 M Tris buffer, pH 9.1, containing 1 mM diflunisal phosphate (DFP), 0.1 M NaCl, and 1 mM MgCl₂] was added to each well and incubated for 10 min at room temperature. Finally, developing solution (100 µl of 1 M Tris base, 0.4 M NaOH, 2 mM TbCl₃, 3 mM EDTA) was added to each well and incubated for 1 min at room temperature. The fluorescence was measured with the CyberFluor 615 Immunoanalyzer (MDS Nordion). Calibration and data reduction were performed automatically.

The detection limit of this immunoassay and the specificity against KLK6 were studied using different ratios of active to inactive protein of all forms (proform and/or degradation products). The sensitivity and specificity of our coating monoclonal antibody, which was used to capture KLK6 in the activity-based probe ELISA assay after probe binding, was also evaluated. Furthermore, the ability to measure the concentration of active (ABP-reactive) relative to total (regular ELISA) KLK6 permitted the calculation of the percentage of the active and ABP-reactive form of KLK6 (KLK6 ABRA-ELISA).

5.2.3 Mass spectrometry analysis of biological fluids

Ovarian cancer ascites fluid (diluted 1:20 in ddH₂O), cerebrospinal fluid, and the cell supernatant were supplemented with 20 µg/ml recombinant active KLK6 (300 ng). Samples were incubated at room temperature for 8 hours (to allow the evaluation of possible protein degradation within the time-frame of the ABRA-ELISA), analyzed by SDS-PAGE, and stained with SimplyBlue™ SafeStain (Invitrogen Canada Inc., Burlington, ON, Canada). Gel bands
were excised and subjected to in-gel trypsin digestion. In brief, the gel was condensed with acetonitrile (Sigma-Aldrich Canada Ltd.) for 10 min at room temperature and the bands were reduced with 10 mM DTT (Sigma-Aldrich Canada Ltd.) for 10 min at 60°C, followed by a 20-min incubation at room temperature. Gel bands were subsequently alkylated with 100 mM iodoacetamide (Sigma-Aldrich Canada Ltd.) for 15 min at room temperature and the gel was treated once more with acetonitrile for 10 min. Finally, proteins were digested with 50 µl of 0.01 µg/ml trypsin (Promega, Madison, WI, USA) in 50 mM NH₄HCO₃. After 16 h of trypsin digestion at 37 ºC, samples were subjected to LC-MS/MS (LTQ Thermo-Finnigan; Thermo Fisher Scientific, Inc), using a protein identification protocol similar to that of Chapter IV. Protein hits were analyzed using the Mascot algorithm (Matrix Science Inc.) and protein probabilities were further evaluated with Scaffold (Proteome Software Inc.). This analysis allowed identification of proteins/peptides in each sample, corresponding to the several molecular masses visualized by SDS-PAGE. Recombinant KLK6 (10 µg) was also subjected to deglycosylation with PNGase F (500 Units; New England Biolabs Ltd), according to manufacturer instructions, followed by SDS-PAGE Coomassie staining and western blot detection. Both samples were also subjected to trypsin digestion and LC-MS/MS protein identification, as described above.

5.3  Results

5.3.1  Quantification of KLK6 in biological fluids using activity-based probe ELISA

The general concept of the KLK6 ABP ratiometric ELISA assay (KLK6 ABRA-ELISA) depends on the quantification of active KLK6, which is described in Figure 24A. The first step shown involves the reaction of Bio-PK with active KLK6. To immobilize the probe-KLK6
complex, we utilized a KLK6-targeted monoclonal antibody that has been previously generated and validated for its sensitivity and KLK6 selectivity in our laboratory (Luo et al., 2006). Detection of the probe-biotin label was performed similarly to the regular KLK6 ELISA assay, incorporating streptavidin-linked detection reagents. In brief, we added a streptavidin-alkaline phosphatase (SA-ALP) conjugate, incubated, washed, and then detected the ALP activity by using diflunisal phosphate as substrate, in combination with terbium chelates and time-resolved fluorescence (Christopoulos and Diamandis 1992; Ferguson et al., 1996; Diamandis et al., 2000d). This assay configuration demonstrated good sensitivity and specificity to quantify active KLK6. Optimal conditions were selected based on the lowest achievable detection limit and best assay linearity and dynamic range.

A typical calibration curve of the active KLK6 ELISA assay is shown in Figure 24B. The detection limit, defined as the concentration of KLK6 corresponding to the fluorescence of the zero calibrator plus 2 standard deviations values (SD), was 0.025 µg/l (about 0.83 pM, considering a molecular weight of about 30 kDa, as per the SDS-PAGE analysis of recombinant KLK6). Within-run and between-run precision was assessed at various KLK6 concentrations between 0.05 and 10 µg/l (1.67-333 pM) and with selected clinical samples. The coefficients of variation (CVs) were between 6-20%.
Figure 24: Active KLK6 ELISA assay; outline of the procedure (A) and calibration curve for detection of active enzyme (B). (A) The schema illustrates the procedure, as outlined in Materials and Methods, for covalently labeling active KLK6 with the biotinylated (B, attached to linker: dark oval) proline-containing ABP (Bio-PK), followed by capture of the biotinylated enzyme with a monoclonal KLK6-targeted antibody (capture Ab). Capture is followed by the generation of the fluorescent signal from the bound streptavidin-alkaline phosphatase (SA-ALP). (B) The graph shows a representative standard curve (logarithmic scale on both axes) of the active KLK6 ELISA assay, using enzyme isolated by the new two-step procedure described in sections 2.2.5 and 2.3.3, and illustrated in Figure 10.
To ensure that the assay specifically measures the active form of KLK6, we mixed inactive and active recombinant KLK6 in different ratios and analyzed the mixtures with the ABRA-ELISA assay. The amount of the active enzyme detected in these mixtures corresponded to the concentration of active KLK6 added originally in the mixture (data not shown). To confirm the specificity of the assay further for active KLK6 we analyzed selected fluids for the levels of active (ABP-reactive) and total (regular ELISA) KLK6: cerebrospinal fluid, ascites fluid from ovarian cancer patients, and cancer cell supernatants (Figure 25).

Figure 25: Levels of KLK6 in biological fluids and cultured cell-derived supernatants, measured as total immunoreactive KLK6 (total KLK6) and as active enzyme (active KLK6). The graphs show the values (each sample shown by an individual symbol) measured for samples of cerebrospinal fluid (CSF; n=20), ovarian cancer-associated ascites (Ascites; n=23), and cell culture supernatants obtained from breast, ovarian, and prostate cancer-derived cell lines (Cell supernatant; n=4). The horizontal bars in each panel show the average values (µg/l immunoreactive or enzymatically active KLK6: 0.03 µg/l = 1 pM, molecular weight = 30 kDa) derived from measurements of all samples in each group. Also shown on the right of each panel is the recovery of active KLK6 (% Recovery of total active KLK6 in supplemented samples relative to an equal amount of supplemented KLK6 in a sample of 6% BSA buffer) for samples to which a known amount of active enzyme was added prior to the conduct of the ABRA-ELISA assay. Note that the there is a log scale for protein concentration used for the left Y-axis.
Our analyses showed that the samples from different sources had comparable levels of immunoreactive KLK6 (mean values: from about 70 to 270 µg/l or approx. 2 to 9 nM). However, the ABP-reactive enzyme levels were substantially lower and there was considerable heterogeneity in the values obtained for the different samples, a number of which had ABP-reactivity below our detection limit. The mean concentration of the active KLK6 was 0.33 µg/l (11 pM) for the CSF (n=20), 0.89 µg/l (30 pM) for the ascites fluid (n=23), and 0.029 µg/l (0.97 pM) for the cancer cell supernatants (n=4). That said, the active KLK6 concentrations ranged from non-detectable to about 8 µg/l (267 pM). We did not find any correlation between the amount of immunoreactive KLK6 and the levels of active enzyme.

In summary, as a common feature of all samples, the majority of the immunoreactive enzyme (approx. 95-99.9%) appeared not to react with the activity-based probe (ABP detection relative to total ELISA), and thus, the majority of enzyme was presumably in the “inactive state” (Figure 25). Ascertaining the molecular forms of the immunoreactive but enzymatically inactive KLK6 remains a topic for our further work.

To validate the ability of our assay to recognize the active form of KLK6 in complex mixtures, we supplemented the above-mentioned biological samples with 7.7 µg/l (257 pM) of recombinant active KLK6. When added first to a clinical sample prior to biotin labeling, the reactivity of KLK6 with the activity-based probe was significantly diminished. According to the calibration curve, the recovery of active enzyme from the supplemented samples ranged from ≥100% of supplemented concentrations (amount of enzyme in KLK6-supplemented samples relative to an equal amount of supplemented KLK6 in a sample of 6% BSA buffer), to levels that were below the detection limit of our assay (Figure 25: right Y-axis). In general, the observed recovery of added active enzyme from CSF and ascites samples was higher than for cancer cell supernatants, which yielded a recovery of less than 50%.
We interpreted these data as an indication that samples, like those obtained from the cancer cell supernatants, contain sufficient inhibitors to bind and inhibit both endogenous active, as well as exogenously added enzymes. Furthermore, the higher than expected recovery of the enzyme in the KLK6-supplemented samples with low endogenous levels of active KLK6 may be the result of further proteinase activation originating from the reservoir of endogenous KLK6 zymogen.

5.3.2 Active KLK6 forms enzymatically inactive complexes in biological fluids

As expected, SDS-PAGE separation and western blot analysis of CSF, MDA-MB-468 cell supernatants, and ascites fluid did not yield a signal at the expected position of KLK6, except for the case of CSF sample (white arrowhead, Figure 26, gel 1). This is due to the relatively low levels of KLK6 in these samples (CSF: 567 µg/l, MDA-MB-468: 13 µg/l, ovarian cancer ascites: 42 µg/l) which lie below the detection limit of our western blot approach. However, the KLK6 western blot analysis did reveal higher molecular mass cross-reactive components migrating in the region of 58 to 62 kDa (upper bands, Figure 26, gel 1). In particular, higher molecular weight bands were strongly visible in the SDS-PAGE gel lanes corresponding to the MDA-MB-468 cell supernatant and ovarian cancer-derived ascites sample.

To verify the presence of enzymatically active KLK6 (expected molecular mass about 30 kDa), we supplemented the three test samples with 20 µg/ml of active KLK6 enzyme. As expected, a strong signal was observed at the position of recombinant KLK6 (~30 kDa; Figure 26, gel 2). To detect enzymatically active KLK6 in the supplemented samples, we used the Bio-PK activity-based probe that covalently attaches its biotin moiety to the active serine of the enzyme (Pan et al., 2006). Addition of the Bio-PK to the KLK6-supplemented samples labeled one principle component that migrated in the gel at the position expected for active KLK6.
(white arrowhead, Figure 26, gel 3). Significantly, labeling by the Bio-PK was not observed in the higher molecular mass regions of the gel (> 80 kDa), where a strong western blot KLK6 signal was observed. Our ABP data implied that the recombinant KLK6 added to the samples and migrating with the higher molecular weight bands under reducing conditions was not enzymatically active. We therefore hypothesized that a proportion of the added recombinant KLK6 was rendered inactive by serpin-related species that were already present in the biological samples. To test this hypothesis, we used a proteomic approach to identify the higher-molecular mass proteins that co-migrated with the KLK6 immunoreactivity in the higher-molecular mass regions of the gels.

**Figure 26: Western blot and ABP analysis of KLK6 in biological fluids: samples supplemented or not with recombinant active KLK6.** Samples of CSF, MDA-MB-468 cell culture supernatant, and ascites fluid were supplemented (gels 2 and 3) or not (gel 1) with 20 µg/mL of recombinant enzymatically active KLK6 (r-KLK6). One set of samples was reacted with the activity-based probe (gel 3) and all three sets of samples, after an 8-hour incubation, were analyzed by polyacrylamide gel electrophoresis (PAGE) under reducing conditions. After transfer of the resolved proteins to Hybond-C Extra (Amersham Biosciences, Pittsburgh, PA, USA) nitrocellulose membrane, protein was visualized either by a western blot procedure (gels 1 and 2) or with the use of streptavidin-alkaline-phosphatase (gel 3: SA-ALP signal) to identify biotinylated protein. The open arrowhead denotes the position in the gels of active recombinant KLK6. The positions of the molecular weight markers (kDa) are shown on the left of the gels.
5.3.3 High molecular mass forms of KLK6 co-migrate with serine proteinase inhibitors

Upon SDS-PAGE analysis, enzymatically active KLK6 yielded only one major coomassie-staining band migrating in the 30-kDa region (Chapter II). In the biological samples supplemented with active recombinant KLK6, this coomassie-staining band was also detected, whereas it was below the level of coomassie-staining detection in the original MDA-MB-468 and ovarian cancer ascites non-supplemented samples (data not shown). Based on the positions of the coomassie-staining bands and the corresponding positions of KLK6 western blot immunoreactivity, gel samples were excised and processed for trypsin digestion and identification by mass spectrometry.

Analysis of the gel fragments obtained from the 20-32 kDa region of the gel as expected identified human KLK6. The tryptic fragment analysis indicated the possible presence of both glycosylated as well as non-glycosylated KLK6, which provided a rationale for the diffuse bands visualized upon KLK6 western blot analysis (Figure 26, gel 2, 1st lane on the right). Trypsin fragmentation and mass spectral analysis of protein in samples obtained from the 50 to 62 kDa region of the gel were found to contain albumin. This area of the gel was therefore difficult to analyze further in terms of its content and identity of potential KLK6 inhibitors. Nonetheless, in the higher molecular weight regions of the gel, where higher molecular mass constituents migrated (above 62 kDa, Figure 27A), the proteomic analysis revealed the presence of KLK6 sequences along with sequences that corresponded to α2-macroglobulin (α2M), α1-antitrypsin (AT), Serpin B3 (B3), and inter-alpha-trypsin inhibitor (ITI). The same proteomic analysis of the samples recovered from the gel from MDA-MB-468 cell supernatant that had not been supplemented with recombinant KLK6 also identified α2M and ITI along with KLK6 in the same gel samples. The sequences of α2M identified indicated that the human protein was present with its unique sequence (DMYSFL̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅̅...
included in a box), and that at least a portion of the α2M detected in the MDA-MB-468 cell supernatants did not come from the bovine serum used to grow the cells. Western blot analysis of the CSF and ascites samples using an anti-α2M probe verified the presence of this protein as for the MDA-MB-468 cell supernatant (Figure 27B). More specifically, the MDA-MB-468 cell supernatant contained a fragment of α2M of about 150 kDa, which was in agreement with the mass spectrometry analysis, as well as the blots shown in Figure 26 (compare Figure 26, gels 1 and 2, with Figure 27B, KLK6 western blot; the α2M complex is indicated with an asterisk).

An in-parallel analysis of deglycosylated recombinant KLK6 by LC-MS/MS suggested that some of the different lower molecular weight forms of KLK6 in our samples may represent proteins with differential N-glycosylation (Figure 28). One of the peptide fragments, the DCSANTTSCHILGWGK contains the potential glycosylation site (N^{132}; underlined), which has been previously reported (Bernett et al., 2002; Bayés et al., 2004). This sequence was identified in the de-glycosylated form of r-KLK6 only (identified 13 times with mascot scores ranging from 18-76). A parallel analysis of the KLK6 in CSF, ascites, and MDA-MB-468 supernatant was partly permitted due to concentration limitation (merely the 30 kDa form of KLK6 was confidently identified in CSF as shown in Figure 28); a western blot of the three samples is included for comparison.
Figure 27: Identification of KLK6 inhibitors in biological samples. (A) CSF, MDA-MB-468 cell supernatant and ovarian cancer ascites fluid (diluted 1:20 in ddH2O) samples were supplemented with 20 μg/mL recombinant enzymatically active KLK6 (r-KLK6: active form, open arrowhead; lower molecular mass form, grey arrowhead) and were subjected to SDS-PAGE separation, under reducing conditions, followed by detection of protein by coomassie stain (lanes 1-4: Panel A). The same series of samples, devoid of supplemented KLK6, were analyzed by coomassie stain (data not shown) or western blot (panel B) with antisera targeted against either KLK6 or α2-macroglobulin (α2M). Gel samples excised with the guidance of the coomassie stained bands, were subjected to trypsin digestion and mass spectral (MS) identification of the tryptic fragments. The arrows to the right of the gels stained with coomassie in Panel A show the components identified by MS analysis at each position in addition to a signal for KLK6: α2M = α2-macroglobulin; AT = α1-antitrypsin; ITI = Inter-alpha-tryps in inhibitor; B3 = serpin B3 or squamous cell carcinoma antigen 1. The asterisks denote positions at which the same components were identified from the gel slices of samples either supplemented or not with r-KLK6. (B) The western blot data show the higher molecular mass forms of KLK6 in relation to the gel positions of the western blot signals for α2-macroglobulin-cross-reactive species. As in Panel A, the asterisk denotes bands detected in samples supplemented or not with KLK6. The positions of the molecular weight markers (kDa), visualized separately, are shown on the left.
5.4 Conclusions

The main outcome of our study is that it is now possible to use combined ABP labelling with ELISA-based detection of total KLK to monitor the proportion of enzymatically active enzyme relative to total immunoreactive KLK present in samples derived from clinical or other sources. In preliminary work, we had established that reaction of the ABP with the enzyme was essentially complete over a time frame of about 4 h (data not shown), and thus, routine labelling in the presence of a molar excess of ABP/enzyme was allowed to proceed for twice this time prior to the antibody capture step. The ELISA was able to validate the recovery of enzyme both
with and without the labelling procedure, confirming that the labelling step *per se* did not effect
the ability of the capture antibody to recover KLK6 protein from the samples. We were thus
confident that the total capture of immunoreactive KLK6 from unknown samples included the
same high proportion of biotinylated enzyme that had reacted with the ABP. We did not observe
significant degradation of KLK6 protein during the time course of the ABP labelling and
ELISA assay (Chapter II, Figure 9, middle gel panel and data not shown).

Our ratiometric approach revealed that the majority of KLK6 quantified by ELISA is
enzymatically inactive in CSF, ovarian cancer ascites fluid, and tumour-derived cell culture
supernatants. This result is in agreement with previous studies reporting that KLK6 exists
mainly as a zymogen in biological fluids (Okui *et al.*, 2001; Hutchinson *et al.*, 2003; Luo *et al.*, 2006). Nevertheless, in all three types of biological samples we were able to label a small but
significant amount of enzyme with the ABP (Figure 25). This relatively low concentration of
active enzyme may not have been detected with the methods that we have previously used.

The low recovery of active enzyme able to react with the ABP reagent in clinical
samples supplemented with pure active KLK6 is notable. We believe that the added enzyme
activity is rapidly quenched by the presence of serine proteinase inhibitors (serpins; Law *et al.*, 2006) known to be present in such samples. This conclusion is supported in large part by:

1. The increased abundance, upon supplementing the samples with recombinant active KLK6,
of KLK6 immunoreactivity that was present in the higher molecular weight regions of the
analytical gels. The ABP labelling procedure indicated that these higher molecular mass
forms of the KLK6 immunoreactivity did not represent active enzyme.

2. The co-migration of KLK6 immunoreactivity under reducing PAGE conditions, along with
known proteinase inhibitors (*e.g.* α1-antitrypsin and α2-macroglobulin), as verified by our
proteomic analysis of components recovered from the higher molecular weight regions of the gels (Figure 27).

A number of studies have already identified the presence of proteinase inhibitors in clinical fluid samples where several kallikrein-related peptidases are expressed. For example, α1-antitrypsin and ITI can be detected in elevated levels in settings of cancer (Kuramitsu and Nakamura, 2006) where KLK6 is also increased (Yousef et al., 2004; Nagahara et al., 2005). Moreover, α1-antitrypsin and α2-macroglobulin have been identified in cerebrospinal fluid, along with KLK6 (Dumont et al., 2004; Yuan and Desiderio, 2005). In previous work using a hybrid antibody approach (selective anti-KLK antibody capture of KLK6 followed by detection of KLK-bound inhibitor with polyclonal antibodies against the inhibitors), it has been possible to identify KLK6 as a complex with α1-antichymotrypsin in fractionated human milk and in ascites fluid from ovarian cancer patients (Hutchinson et al., 2003). However, other potential inhibitors (e.g. α2-macroglobulin or α1-antitrypsin) were not detected in the fractionated samples. *In vitro* studies have also shown that KLK6 can bind to α2-antiplasmin and antithrombin III (Magklara et al., 2003).

The proteomic detection of α1-antitrypsin and α2-macroglobulin co-migrating with KLK6 in our samples, suggests that complexes with these two inhibitors are also possible. In summary, we present a hypothetical model of KLK6 inhibition in the biological fluids we surveyed (Figure 29), which merits further testing with the hybrid antibody approach, as previously used (Hutchinson et al., 2003). The already published KLK6 data that are in accord with our new results are paralleled by the identification of complexes of KLK5 with inhibitors, such as α1-antitrypsin and α2-macroglobulin in serum and ovarian cancer ascites fluids (Yousef et al., 2003b).
Figure 29: Regulation of KLK6 activity in biological fluids. A model of the different forms of KLK6 in CSF, ovarian cancer-derived ascites fluid, and supernatant from human cancer cell lines is presented in the upper panel. Based on this model, in the lower panel we suggest what would be the fate of active KLK6 in these fluids, in the case of a sudden concentration increase of the active enzyme. In this model we have mainly included the glycosylated enzyme since this was identified by SDS-PAGE and MS in our samples. However, the role of presence or absence of glycosylation in regulation of KLK6 activation, degradation or interaction with inhibitors and substrates remains to be investigated.

Our data obtained using the activity-based probe show that, even if able to form enzymatically inactive complexes with added KLK6, the inhibitors were not sufficient to entirely block the activity of the added enzyme visualized by ABP labelling (Figure 26). However, the complex of α2-macroglobulin with KLK6 may be of special interest, since this high molecular weight inhibitor, may be able to regulate the clearance of KLK6 by endocytosis,
as well as protect the proteinase from untoward degradation or inhibition by serpins. In principle, a proteinase, bound to a proteinase inhibitor that can block its enzymatic activity while preserving the integrity of its active site, can subsequently be released in an active form when needed. This situation has been described for another serine proteinase, tissue plasminogen activator (tPA), which also forms a complex with α2-macroglobulin in patients with acute myocardial infarction (Ieko et al., 1997). Although not reactive with the activity-based probe, the KLK6-α2-macroglobulin complex might be able to cleave selected small peptides but not intact protein substrates. This kind of restricted reactivity against small substrates has been reported for the complex formed between another kallikrein-related peptidase, KLK3 (also known as prostate-specific antigen, PSA), and α2-macroglobulin (Christensson et al., 1990).

Although the coomassie stain revealed one principal protein in our active KLK6 preparation (Figure 27A, right panel), a micro-heterogeneity of KLK6 was detected by western blot analysis, particularly in the lower molecular mass region (20-30 kDa, Figure 26, gel 2, and Figure 27B). That said, the ABP labelling showed only one principal enzymatically active species (Figure 26). Although the western blot sample may have been overdeveloped to visualise the lower molecular mass species (20-30 kDa, Figure 26, gel 2), the proteomic analysis indicated the presence of a glycosylated form of KLK6 recovered from the gel. Thus, the diffuse staining of KLK6 can be attributed to its differential glycosylation; these lower molecular mass species detected by the western blot analysis did not appear to react with the activity-based probe (Figure 26, gel 3) and, therefore, were very likely enzymatically inactive. The precise identity of these lower molecular mass species has yet to be determined. Furthermore, the role of glycosylation for the enzymatic activity and turnover of KLK6 remains a topic for further study.
Chapter VI.

Discussion
DISCUSSION

The results of Chapters II to V dealt with the role of kallikrein-related peptidases against the proteinase-activated receptors. We described the production of active KLKs, amongst which the novel three-step production of active KLK6 from a mammalian source. We subsequently used these active enzyme preparations to investigate their interaction with PARs in vitro, ex vivo, and in vivo (Chapters III and IV). Finally, we aimed to discover the physiological range of active KLK concentration in biological fluids and clinical samples. The conclusions derived from these studies were individually outlined at the end of each Chapter. The following discussion, takes into account the fact that KLKs can now be considered as highly potential physiological agonists for PARs, while dealing with additional conclusions surrounding these findings.

6.1 Kallikrein-related Peptidases as Hormone-bearing Messengers

The classical definition of a hormone requires that the candidate protein is synthesized in one tissue, from where it enters the blood circulation to control the action of another distant tissue. These characteristics of secretin, led Bayliss and Starling (1902) to use the term ‘hormone’, possibly inspired by the Greek verb for ‘excite’ or ‘arouse’ - ormao (Henderson, 2005). By that definition, several other proteins can qualify as hormones. For example, amongst proteinases, thrombin is synthesized in the liver and circulates through the bloodstream to act upon its target tissues (e.g. endothelium and platelets) (Barnhart, 1965).

In addition, from the functional perspective, proteinases can have hormone-like features. Apart from their work in protein catabolism, which is well-appreciated, several observations have led to the conclusion that proteinases can mimic the hormonal action of insulin. For
example, work in the mid-1960s, showed that pepsin and chymotrypsin could promote glycogen formation in a rat diaphragm preparation (Rieser and Rieser, 1964; Rieser, 1967). Soon thereafter it was observed that trypsin, could as well stimulate glucose oxidation and inhibit lipolysis, similarly to insulin (Kono and Barham, 1971). Furthermore, studies on the proteolytic enzymes thrombin and trypsin revealed that these proteinases, like insulin, can stimulate mitogenesis in cultured cell systems (Burger, 1970; Sefton and Rubin, 1970; Chen and Buchanan, 1975). Thus, the ability of proteinases to fulfill the criteria expected of a hormone and to trigger ‘hormone-like’ responses in target tissues have been known for almost half a century, but it is only recently that the mechanisms responsible for the cellular actions of a number of proteinases have come into sharper focus.

Kallikrein-related peptidases are synthesized in several tissues and, by means of the bloodstream and other biological fluids, travel in surrounding and distant cells to exert their biological actions (Borgoño et al., 2004; Borgoño and Diamandis, 2004). Moreover, it has been established that they can induce the proliferation of several cells, such as keratinocytes (Klucky et al., 2007) and prostate cancer cells (Klokk et al., 2007). Amongst the members of the KLK family, kallikrein 1 can also have mitogenic effects, through the generation of active kinin peptides from their kininogen precursor (Roberts and Gullick, 1989; Bhoola et al., 1992). The main outcome of our study was that the kallikrein-related peptidases can signal via proteinase-activated receptors (Chapter III and IV), a family which has been implicated in several pathways of cell signalling (Steinhoff et al., 2005); therefore, one can now consider these receptors as means via which KLKs, like hormones, can transmit their chemical messages in an autocrine or paracrine manner.
6.2 Activity-based Probes as Tools to Detect Serine Proteinase Activity

Despite the conclusions derived out of our pharmacological approach (Chapter III) and the strong indications of proteinase-activated receptors being targets of kallikrein-related peptidases in vivo (Chapter IV), we could still not establish the role of KLKs as physiological micro-environmental modulators. The missing piece from our puzzle was to define what the physiological concentrations of active KLKs are in vivo. In other words we wanted to determine if the high KLK levels utilized during our pharmacological studies were within the physiological range of their action. For that purpose we turned into probes of enzyme activity.

For the past few years enzyme probes have been developed as a major tool to monitor protein activity within and outside the cells. For example, several fluorescence-based imaging tools have allowed for the functional study of proteins in several settings (Baruch et al., 2004). Many of these methods suffer in terms of the selectivity of probes for a specific enzyme target; however, recently chemically modified molecules have been successfully recruited to restrict the group in one class of enzymes (e.g. trypsin-like proteolytic enzymes; Pan et al., 2006). These probes are called activity-based probes (ABPs) and have several advantages over the classical substrate-type probes in that they can be designed to be highly specific for distinct subfamilies of proteinases.

Currently, activity-based probes with different chemical properties have been synthesized (Sadaghiani et al., 2007). For example, radiolabeled probes exhibit high sensitivity and broad sample applicability; nonetheless, they are mainly used for gel-based detection of enzyme activity. Fluorogenic tags allow the imaging of proteases in a wide range of samples and have the advantage of being applicable both in vitro and in vivo (Sadaghiani et al., 2007). Finally, biotinylated probes are generally limited to in vitro profiling applications and are
primarily used for enrichment of samples, which can be later subjected to identification of target enzymes by mass spectrometry (Sadaghiani et al., 2007).

Despite the high synthesis cost of the fluorogenic activity-based probes (Sadaghiani et al., 2007), a widely applicable modification of this group, the so called quenched activity-based probes, has been recently developed (Blum et al., 2005; 2007). Due to their high selectivity, decreased background fluorescence release, and efficient cell-permeability, these probes may allow imaging of specific proteinase activity at considerably earlier time points than can be used for substrate-based methods or non-quenched ABPs. Therefore, it has been suggested that they may have the potential to be used in the context of a live cell or whole organism, such as in intra-operative procedures in which rapid production of specific signal is required. Unfortunately, thus far, quenched activity-based probes have only been developed against cysteine proteinases (Blum et al., 2005; 2007).

The different biotinylated probes can also find a wide range of applications. Biotin is a cheap and efficient reagent to use for chemical synthesis of probes that can be conjugated to avidin for detection or analyses purposes (Fonović et al., 2007). Nevertheless, biotin has low cell permeability properties, which prevents its use for in situ or in vivo applications (Sadaghiani et al., 2007). A great disadvantage is also the high level of endogenously biotinylated proteins that may be purified by the avidin resin. Furthermore, the high affinity interaction of biotin with avidin requires the use of harsh denaturing conditions for protein elution, with buffers that may not be compatible with mass spectrometric analysis. To bypass this problem, additional steps are needed for buffer removal, which may result in significant loss of sample (Fonović et al., 2007). Additional disadvantages, when direct digestion of the avidin-conjugated proteins is used, are the contamination by avidin and the increased false positives as a result of non-specific binding of proteins to avidin (Fonović et al., 2007). To confront some of the limitations
of biotin, chemically cleavable biotinylated activity-based probes have been synthesized (Fonović et al., 2007). These probes contain a cleavable linker, such that a mild chemical treatment is sufficient to release the enzymes from the probe-enzyme immobilized complexes.

Several of the activity-based probes have been coupled with proteomics to develop methods for activity-based probe profiling of biological samples (ABPP) (Jessani et al., 2005; Madsen et al., 2006; Paulick and Bogyo, 2008). Even though it has been noted that the majority of these studies have relied on gel-based proteomic analysis, which may be difficult to automate or used to distinguish among proteins coming from highly related protein species (Okerberg et al., 2005), these methods made a major breakthrough for the development of functional proteomics analysis. The greatest applicability of functional proteomics has thus far been the profiling of enzymes involved in cancer-related samples (Jessani et al., 2005; Madsen et al., 2006; Paulick and Bogyo, 2008). In one of these studies, urokinase plasminogen activator was identified as a key player of tumour intravasation, a significant process of the early events of metastasis (Madsen et al., 2006).

For this present study we selected to work with biotinylated activity-based probes. Since kallikrein-related peptidases are all secreted proteins, we assumed that this type of activity-based probes would be the most cost-efficient way to target KLK activity, without being restricted by the potential disadvantages of the biotin group. The general structure of our probes contains the classical ABP functional groups: a phosphonate reactive group (which modifies an active-serine residue within the active site of any trypsin-like proteinase), a linker region, and a biotin tag to visualize the modified enzyme (Pan et al., 2006; Paulick and Bogyo, 2008). The interaction between the probe and the enzyme is characterized as a suicide inhibitor mechanism, such as the inhibitor is covalently and irreversibly bound to the proteinase. This reaction mimics
the interaction of serine proteinases with many of their inhibitors, also known as serpins (Law et al., 2006).

6.3 Detection of Active KLKs in Biological Fluids

Utilizing the activity-based probes of serine proteinase activity and taking advantage of the high efficiency of thermolysin as an activator of KLK6 we succeeded in developing a novel method for production of active KLK6 from HEK-293 cell supernatants. Again with the help of these probes, we sought to develop a very sensitive method for the quantification of active KLKs in biological fluids using KLK6 as a paradigm of one of the most widely expressed and functionally active members of the family (Chapter V, Figures 24-25). To the best of our knowledge, this is the first study that combines the high throughput and high sensitivity of an ELISA with the advantages of an ABP analysis. Furthermore despite our previous reports of KLK activity in biological fluids, this is the first attempt to define the ratio of active to inactive KLK6 enzyme in these fluids.

With the combined proteomic-ABP labelling analysis of CSF, cultured cancer cell supernatants, and ovarian cancer-associated ascites fluid we showed that only a low proportion (approx. 0.1-5% detectable only with the ABRA-ELISA) of the immunoreactive KLK6 in such samples represents active enzyme, which is not complexed to inhibitors. However, even though the absolute concentrations of active enzyme in our clinical and cell-derived samples were low (~0.04-8 Units/l of trypsin-like activity; see Materials and Methods of Chapter V), the total amount of enzyme in the samples, if generated in a restricted environment, would in principle be sufficient to cause cell and tissue signals by cleaving and activating PARs (Chapter III) or via other mechanisms. Furthermore, the total levels of immunoreactive KLK6 in all the samples
we surveyed remained high, leaving a possibility for an extracellular stimulus (e.g. increase in pH or increase in the levels of an activating proteinase) to trigger the generation of active KLK6 from the zymogen reservoir.

Therefore, the active enzyme produced by tissues at a localized site could be present in concentrations able to cleave extracellular matrix molecules or myelin-related proteins, all of which have been established as targets of KLK6 in vitro, as well as regulating the PARs. This proteolytic activity could, in principle, contribute to disease pathogenesis (Borgoño and Diamandis, 2004).

6.4 Role in Physiology and Pathophysiology

The ability of kallikrein-related peptidases to signal via proteinase-activated receptors is of particular interest because of the wide distribution of KLKs in many human tissues, their up-regulation by sex steroid hormones, and their increase in a variety of pathological conditions (Borgoño et al., 2004; Borgoño and Diamandis, 2004). The three KLKs included in our study, KLKs 5, 6, and 14 are widely distributed in the body (Shaw and Diamandis, 2007 and sections 2.1.1, 2.1.2, 2.1.3), representing prime candidates as physiological regulators of PAR2 in many settings, adding to the ability of other serine proteinases like trypsin, factor VIIa/Xa, and matriptase to activate PAR2 (Steinhoff et al., 2005). Unlike human mast cell tryptase, another potential physiological activator of PAR2, KLKs were not restricted in their ability to activate the receptor by the glycosylation site in proximity to the cleavage-activation target of the enzyme (Compton et al., 2001; 2002a,b). Thus, in contrast with tryptase, KLKs 5, 6, and 14 were able to reduce vascular tension via PAR2 activation in an intact rat and mouse aorta tissue assay.
In general, unlike pancreatic trypsin, with its somewhat limited tissue distribution, and unlike tryptase, with its restricted ability to activate PAR2, the widely distributed tissue KLKs via PAR2 more specifically and via PAR1, PAR2, and PAR4 in general would, in principle, be able to effect a large number of tissues in the body, ranging from platelets to vasculature (Steinhoff et al., 2005; Ramachandran and Hollenberg, 2008). This potential ability of KLKs to regulate tissue function via the activation of PARs can now be added to other proposed mechanisms (e.g. degradation of extracellular matrix, activation of growth factors or generation of bradykinin, and processing of growth factor-binding proteins) believed to explain the complex biological actions of these serine proteinases in a variety of pathophysiological settings, such as tumour survival, metastasis, and angiogenesis (Borgño et al., 2004; Borgño and Diamandis, 2004).

Apart from receptor activation, the enzymatic dis-arming/inhibition of a PAR may be equally important. By preferentially activating human platelet PAR4 (Figure 20) while dis-arming PAR1 (Figures 17 and 20), kallikrein-related peptidase 14 would promote the release of platelet endostatin and suppress the release of platelet vascular endothelial growth factor (Ma et al., 2005). The preferential activation of PAR4 versus PAR1 by KLK14 mirrors the action of plasmin on human platelets, wherein plasmin can both activate and dis-arm PAR1 (Kuliopulos et al., 1999; Quinton et al., 2004). Whether PAR1 would be activated or dis-armed by KLK14 (Figure 17) would depend on the ambient concentrations of the enzyme at a given location and on the relative rates of cleavage at the tryptic/chymotryptic cleavage sites. Furthermore, by selectively activating PAR2 and PAR4, while dis-arming PAR1, KLKs in general and KLK14 specifically could contribute to processes related to the inflammatory response (Vergnolle et al., 2001b; Vergnolle et al., 2002; Hollenberg et al., 2004; Vergnolle, 2004). These PAR-targeted actions of the kallikrein-related peptidases would add to the known ability of at least one
member of the family, KLK1, to generate inflammatory/nociceptive bradykinin peptides from kininogen (Bhoola et al., 1992).

The role of KLK-derived activation or dis-arming of PARs may have applicability in different tissues, where distinct groups of KLKs are expressed (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Shaw and Diamandis, 2007). For example, KLKs 6 and 8 are widely expressed in the brain (Yousef et al., 2003c) at sites of PAR\textsubscript{1} and PAR\textsubscript{2} expression (Noorbakhsh et al., 2003) and may therefore serve as potential agonists of these receptors in the central nervous system in normal or pathological settings. Other possible pathophysiological roles for localized kallikrein-related peptidases may be found in the skin, where PARs and KLKs are co-expressed (Santulli et al., 1995; Komatsu et al., 2003; Steinhoff et al., 2005) either in normal or pathological settings, such as psoriasis (Ekholm and Egelrud, 1999; Kuwae et al., 2002; Iwakiri et al., 2004). Further, uncontrolled KLK action in the skin can also lead to desquamation (Caubet et al., 2004), a setting in which PARs may be inappropriately over-activated.

Another situation in which KLKs may represent endogenous PAR modulators is in tumourigenesis, a field, which will be discussed in detail in the next section. There is now an extensive literature dealing with changes in both the PARs (Steinhoff et al., 2005) and the KLKs in the setting of cancer, including cancers of the ovary, breast, colon, pancreas, and prostate (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Yousef et al., 2004; Obiezu and Diamandis, 2005; Yousef et al., 2005). These changes in the levels of kallikrein-related peptidases can be correlated with either a favourable or unfavourable prognosis (Borgoño et al., 2004; Obiezu and Diamandis, 2005; Yousef et al., 2005). It will be essential in these settings to determine the proportion of the enzyme that is catalytically active.

In summary, our data add the PARs to the list of KLK targets that may explain the signalling properties that this enzyme family can have in many settings. We suggest that KLKs
may have a dual role during cancer onset and progression, similar to the metalloproteinases (Boire et al., 2005). Like the KLKs, the metalloproteinases can cleave proteins of the extracellular matrix (e.g. collagen, fibrinogen, and metalloproteinases) or they can activate receptors like the PARs, both related to tumour growth, metastasis, and survival. Via the PARs, the KLKs can potentially signal by a variety of G-protein-coupled signalling pathways (G_q, G_i, and G_{12/13}). PAR signalling has been further implicated in a variety of (patho)physiological processes, including the regulation of vascular and gastric smooth muscle contractility, delayed immune response, inflammation, nociception, as well as angiogenesis, cell apoptosis, adhesion, proliferation, metastasis, and invasion (Macfarlane et al., 2001; O’Brien et al., 2001; Vergnolle et al., 2001b; Noorbakhsh et al., 2003; Steinhoff et al., 2005). The effects that kallikrein-related peptidases may have in any situation will depend on the spectrum of KLKs present, the levels of the active free enzyme, and the availability of PARs that may be expressed in any specific environment. In this regard, the kallikrein-PAR axis may be seen as a fruitful therapeutic target for a number of disease states.

6.5 Clinical Utility of Active KLKs in Inflammation and Cancer

Conclusively, our past and current data point to active KLKs as indices of inflammation and cancer. There has long been a debate as to whether inflammation and carcinogenesis share important features and should therefore be considered as biochemically and biophysically similar pathologies. This idea was first introduced in 1863 by Rudolf Virchow, who demonstrated the presence of leukocytes in neoplastic tissue (Macarthur et al., 2004). The hypothesis has been revisited by many researchers, and currently there are several publications, which speak in favour of a relationship between chronic inflammation and cancer with multiple
cross-points along their mechanisms (Coussens and Werb, 2002; Rogers and Fox, 2004; Macarthur et al., 2004; Allavena et al., 2008a,b).

More specifically, experimental, clinical, and epidemiological studies have shown that persistent inflammation can contribute to cancer development and offer predisposition to carcinogenesis (Macarthur et al., 2004; Allavena et al., 2008a,b). In contrast to acute inflammation, chronic inflammation is characterized by infiltration of damaged tissue by mononuclear cells, such as macrophages, lymphocytes, and plasma cells. Examples of chronic inflammatory conditions related to cancer include chronic pancreatitis, which has been linked to pancreatic cancer, as well as inflammatory bowel disease, which can increase the risk of colorectal cancer (Macarthur et al., 2004). Similarly, infection-acquired inflammation is involved in the pathogenesis of a significant number of tumours (Rogers and Fox, 2004; Allavena et al., 2008a,b). A few examples are the chronic infection by hepatitis, which can lead to hepatocellular cancer, as well as the infection by human papilloma virus (HPV), certain strains of which can cause anogenital cancer (Macarthur et al., 2004).

Furthermore, the extracellular tumour niche can be characterized by the presence of at least one inflammatory component, similarly to inflamed tissues (Macarthur et al., 2004; Allavena et al., 2008a,b). These components may include tumour necrosis factor (TNF) or interleukin-1 (IL-1), which are critical for inflammatory response. It has been suggested that an intrinsic pathway of tumour-related inflammation (neoplastic transformation of tumour cells), as well as an extrinsic pathway (in tumour-infiltrating leukocytes), are in effect in the tumour micro-environment and can both contribute to tumour progression by increasing the availability of bioactive molecules (Allavena et al., 2008a,b).

Amongst the components of the immune system, tumour-associated macrophages seem to be the key regulators of the link between inflammation and cancer (Allavena et al., 2008b;
Marx, 2008). Macrophages naturally form part of the body’s powerful defence against tissue injury and pathogen invasion; however, in the settings of cancer, they can express growth factors, proteinases, and immune mediators, which can promote cell survival or suppress adaptive immunity, increase angiogenesis, and initiate tissue destruction to facilitate tumour invasion and metastasis (Macarthur et al., 2004; Allavena et al., 2008b).

Interestingly, it has been reported that many, if not all of the immune compartments, including peripheral blood leukocytes and cultured Jurkat T cells, can express several members of the KLK family, such as KLKs 5, 6, and 14 (Scarisbrick et al., 2006). Furthermore, it has been reported that KLK6 is abundantly expressed by T cells, as well as macrophages, within multiple sclerosis lesions (Scarisbrick et al., 2002). Therefore, in cases of chronic inflammation, immune cells recruited in the site of injury may contribute to rapid increases in local KLK activity.

On the other side of the coin, several proteinases are expressed by the tumour cells themselves. Although much attention has been given to the potential roles of matrix metalloproteinases (MMPs) in either promoting or inhibiting cancer spread (Seiki, 2003; López-Otín and Matrisian, 2007), serine proteinases are also of considerable importance because of their abundance in the tumour micro-environment. For example, many of these serine proteinases and metalloproteinases have been implicated in the pathology of ovarian cancer (Stack et al., 1998; Camerer, 2007a), such as the urokinase plasminogen activator (uPA) (Mazar, 2001; Choong and Nadesapillai, 2003; Duffy and Duggan, 2004), a serine proteinase, which converts plasminogen to plasmin.

Our work focuses on the kallikrein-related peptidase family of serine proteinases, which are highly abundant in various types of cancer. Extensive literature has suggested a role for KLKs in diagnosis, prognosis or monitoring of several types of cancer, including ovarian,
prostate, and breast (Borgoño et al., 2004; Borgoño and Diamandis, 2004). For example KLK3 or PSA is an established biomarker for monitoring prostate cancer patients. Another member of the family, KLK6 is a putative biomarker of ovarian cancer prognosis and response to treatment. However, no kallikrein-related peptidase, apart from PSA, or any combination of members of the family, has thus far served as an optimal marker with clinical utility for cancer.

Since, KLKs are serine proteinases with trypsin- or chymotrypsin-like activity it is intriguing to hypothesize that the active enzymes play a role in tumour invasion, metastasis, and progression of disease, a great part of which may be facilitated via PAR signalling. Nevertheless, the levels of the active enzymes, KLKs and several other serine proteinases, in tumour extracts and cancer-related biological fluids are still unknown, mainly due to lack of adequate and time-efficient methods for targeted quantification of a specific enzyme’s activity.

The new ratiometric assay approach described in our study (Figure 24) can identify both active and inactive forms of KLKs in clinical samples (Figure 25). Therefore, this method can allow us to establish the physiologically relevant concentration of active KLKs in biological samples, as well as serve as a novel diagnostic tool to identify the active form of KLKs in pathophysiological settings. This approach resembles the free-to-total protein measurements of serum kallikrein-related peptidase 3 or PSA, which has significantly increased the clinical value of this KLK for discrimination of prostate cancer patients with low PSA values from the patients with benign prostatic hyperplasia (Abrahamsson et al., 1997; Catalona et al., 1998, Catalona et al., 2000). Interestingly, the percentage of free PSA has been found to be lower in serum samples from patients with prostate cancer, where a lower percentage of free PSA may be associated with a more aggressive form of prostate cancer (Arcangeli et al., 1996; Carter et al., 1997; Arcangeli et al., 1998; Pannek et al., 1998; Catalona et al., 1998).
As alluded to above, the total amount of kallikrein-related peptidase 6 and other KLKs in our biological samples, if generated in a restricted environment, such as the ascites fluid within the abdominal cavity, would in principle be sufficient to signal via receptors, like the proteinase-activated receptors (PARs), cleave extracellular matrix molecules, activate other proteinases or activate latent growth factors, all of which have been implicated in the process of carcinogenesis, angiogenesis, and tumour survival (Borgoño et al., 2004; Borgoño and Diamandis, 2004; Chapter III). It is interesting to note that a mechanism of dys-regulated proteinase-activated receptor turnover has been proposed to be in effect in cancer, expanding the frequency and number of signals that a cancer cell can accommodate upon proteinase activation (Arora et al., 2007).

We hypothesize that measurement of the proportion of active KLKs in clinical samples will provide information that is complementary to the current KLK ELISA measurements now being used as biomarkers for disease diagnosis and prognosis. In the future, our method may serve as a model for identification of other classes of proteolytic enzymes in biological samples. More importantly, our approach has the potential to develop in a high throughput, automated fashion to facilitate measurements of more than one proteinase in patient samples. We anticipate that this approach will also allow the development of personalized cancer therapeutics, since distinct proteinases may be expressed in their active form in different sets of patients.

Finally, because GPCRs are tractable drug targets, the mechanisms by which these receptors and their associated proteins impact cellular transformation and metastasis might lead to novel cancer therapies (Spiegelberg and Hamm, 2007). Under this idea, efforts have been focusing on developing compounds to block either the proteinase-activated receptors or their activating proteinases (described in detail in the introductory section 1.3.6). Thus far, efforts
have been successful for the development of effective PAR1 antagonists, but several promising data on molecules targeting other PARs have also been reported (Hansen et al., 2008a).

In the future, it may be of unique interest to consider the nuclear and cytosolic receptors as proteinase targets in cancer. A member of the proteinase-activated receptor family or any other receptor for proteinases has not as yet been localized within the cellular compartments. Nevertheless, there are a number of intracellular proteinases from several families, including one isoform of the KLK family (Korkmaz et al., 2001), which makes the existence of an intracellular proteinase-activated receptor as target of these proteinases look like an intriguing hypothesis.

6.6 Inhibitors as Modulators of Untoward KLK Activity

In Chapter V we supplemented our tumour-related samples with recombinant active KLK6 to determine the reason for the low levels of ABP-reactive KLK6 in these biological fluids. We showed that the increase in enzyme activity of KLK6 was rapidly quenched. The presence of serine proteinase inhibitors in these samples can provide a rationale for this observation, since these inhibitors can rapidly form enzymatically inactive complexes with the catalytically active recombinant KLK6 to regulate its activity.

The ability of KLK6 to form enzymatically inactive complexes with a variety of non-selective proteinase inhibitors [e.g. α2-macroglobulin, α1-antitrypsin, Serpin B3, and inter-alpha-trypsin inhibitor (ITI)] suggests that the micro-environment in which the KLKs are produced and activated may be regulated in favour of strictly limiting the duration and extent of action of the enzymes. Thus, the biological actions of the KLKs in the environment of tumours, as in the case of matrix metalloproteinases and their tissue inhibitors (TIMPs; Nagase et al.,
will depend on the balance between enzyme activation and the abundance of their accordant inhibitors, which may also have signalling properties themselves (Stetler-Stevenson, 2008). In other words, measurements of the abundance of serine proteinase inhibitors capable of attenuating KLK action in the settings of cancer or other pathologic conditions can be seen as the essential ‘other side of the coin’ in understanding the pathophysiology of the KLK family of enzymes.

These serine proteinase inhibitors (serpins; Borgoño et al., 2004; Borgoño and Diamandis 2004; Luo and Jiang, 2006) can play key pathophysiological roles, as it has been found in the setting of Netherton syndrome. In this syndrome, a defect in SPINK5 gene, encoding for a Kazal-type 5 serine proteinase inhibitor, can lead to a KLK-mediated ichthyosiform skin disease (Chavanas et al., 2000; Komatsu et al., 2008). Another great example of proteinase-inhibitor disequilibrium can be found in the settings of α1-antitrypsin deficiency; a critical mutation in α1-antitrypsin gene results in retention of the inhibitor within the hepatocytes, limiting its inhibitory effect against proteinases, such as neutrophil elastase in the lung (Stoller and Aboussouan, 2005). This condition is clinically characterized by liver disease and early-onset emphysema.

6.7 **A Physiological Role for Activation of KLK6 by Bacterial Proteinases like Thermolysin**

It is becoming widely appreciated that, in the setting of infections by pathogenic organisms (viruses, bacteria, parasites), proteinases coming from the pathogens themselves or induced in target tissues in the presence of the pathogens can regulate their host environment. An example recruited from the field of proteinase receptor signalling is the house dust mite
Dermatophagoides pteronyssinus; cysteine and serine proteinases released from this pathogen (mite allergens Der p1, Der p3, and Der p9) have been reported to activate PAR\textsubscript{2} in human airway epithelial cells causing release of proinflammatory cytokines from the epithelium (Sun et al., 2001; Asokananthan et al., 2002).

Inspired by our finding that KLKs, such as KLK6, can regulate cell signalling via targeting of the PARs, it is intriguing to hypothesize that bacterial pathogens can manipulate the KLK content of the host micro-environment to regulate cell signalling. This hypothesis, even though not studied in detail for the KLKs, has been investigated in a murine model of bacterial-induced colitis (Hansen et al., 2005). In this study, Citrobacter rodentium infection induced the production of PAR\textsubscript{2}-activating serine proteinases that in turn activated PAR\textsubscript{2} in a mouse model of infectious colitis. In the infected luminal fluid, several active serine proteinases of the host were identified, including granzyme A, kallikrein B, and trypsinogen 16 from the Mus musculus genome, which could be responsible for regulating the colonic inflammatory response.

The proteinase thermolysin, which we used for activation of our KLK6 preparation, is also of bacterial origin. It was originally isolated from the gram-positive bacteria Bacillus thermoproteolyticus and exhibits zinc-dependent enzyme activity (Häse and Finkelstein, 1993; Barrett et al., 2004). In our study, thermolysin efficiently cleaved within the site required for the KLK6 pro-peptide removal (…K–LV…) allowing for the rapid activation of KLK6 zymogen. Several other bacterial species with high pathogenicity, such as the Streptococcus and Staphylococcus spp., can secrete metalloproteinases homologous to thermolysin (Häse and Finkelstein, 1993). Thus, in principle, any metalloproteinase with “thermolysin-like” activity may be able to activate the pro-form of human proteinases, such as the KLKs, unleashing their proteolytic and signalling power in the settings of infection.
6.8 Conclusions in Brief

To summarize, the main findings outlined in this thesis support the hypothesis that kallikrein-related peptidases can signal via proteinase-activated receptors. More specifically, we have shown that:

1. KLKs can activate/dis-arm PARs with different potencies.
2. PAR2 fragments are released when breast cancer-derived cells are treated with KLKs.
3. KLKs can induce mouse inflammatory response.
4. A small but significant amount of active KLKs is present in biological fluids.

Our results indicate that active KLKs can single out PARs as targets in the settings of cancer and inflammation \textit{in vivo}. Further, the amounts of KLKs, released by the tumour or stroma cells in biological fluids may be a significant reservoir of active proteinases capable of regulating signalling in tumour, inflammatory or other neighbouring cells when triggered to activation.

6.9 Future Directions

In the future, it will be important to evaluate the potential inflammatory and nociceptive roles that the KLKs may play \textit{in vivo}. For this work, PAR-null animals should be recruited to investigate the role of these receptors in KLK-mediated inflammation. More, specifically the inflammatory response of PAR1, PAR2, and PAR4-null animals to KLK5, KLK6, and KLK14 treatment should be evaluated, accompanied with measurements of markers of inflammatory response (\textit{e.g.} myeloperoxidase activity). The same animal models should be utilized to evaluate the nociceptive response to sub-inflammatory doses of KLKs. Further, it will be essential to determine the proportion of all the kallikrein-related peptidases that are catalytically active, both
in the rodent models of inflammation, as well as in human inflamed tissues, tumour cells, and related fluids, or in samples derived from patients experiencing chronic inflammation and pain.

It would also be of clinical importance to establish a high throughput approach that will allow for the rapid quantification of all active proteinases (e.g. serine, cysteine, metalloproteinases) in clinical samples. This approach may serve as a novel tool to simultaneously identify the active form of kallikrein-related peptidases or other active serine proteinases in several settings. More importantly, it will allow us to gain insights into the network of proteinases that may be critical during the inflammatory response or essential for tumour survival and progression, so as to generate appropriately customized therapeutic modalities.

Finally, it would be of major importance to delineate the signalling pathways that act downstream of KLKs and PARs. The different kallikrein-related peptidases may have differential effects on cells and tissues via the activation of multiple signalling pathways. These effects may additionally depend on the presence of several co-factors. Identification of these distinct pathways will also contribute to the selection of therapeutic targets for treatment of patients suffering from inflammatory diseases or cancer.
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