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Proteases from seafood as activators of protease-activated receptor-2 in airway epithelial cells

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Contents

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At the start of a thesis in molecular biology that includes extensive work with live cell cultures, few advices can prepare a PhD student for all the mistakes you inevitably will make. In lack of the “magic touch” senior researchers seems to possess combined with assays involving the use of cell cultures that seems to display autonomous behaviour, positive results appear only every solar eclipse. I have been so fortunate to work in an environment that has allowed me to make all my own mistakes and I am confident this has made me wiser over the years.

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Presented papers

Paper I

Bang B, Larsen M, **Larsen AK**, Aasmoe L. (2009). Proteases from salmon stimulate IL-8 in airway epithelial cells. *J Toxicol Environ Health* A. 72 (9): pp 571 – 573

Paper II

Larsen AK, Seternes OM, Larsen M, Aasmoe L, Bang B. (2008). Salmon trypsin stimulates the expression of interleukin-8 via protease-activated receptor-2. *Toxicol Appl Pharmacol* 230 (3): pp 276 – 282

Paper III

Larsen AK, Kristiansen K, Seternes OM, Bang B. Molecular modelling of trypsin from the king crab (*Paralithodes camtschaticus*). *Manuscript – short communication*

Paper IV

Larsen AK, Seternes OM, Larsen M, Kishimura H, Rudeskaya G, Bang B. Purified sardine and king crab trypsin stimulate IL-8 secretion and NF-kB activation, at least partly, via PAR_2, but displays individual differences in transformation of the NF-kB-signal. *Manuscript*.

Introduction

Airway reactivity in seafood industry workers

Workers in the Norwegian salmon industry show enhanced occurrence of respiratory symptoms from both the upper and lower parts of the airways [2, 3]. Frequent sneezing, running nose, sore throat, cough with phlegm, wheezing, and shortness of breath are all symptoms that are found to display increased frequency in salmon workers compared to control groups. Although the prevalence of asthma symptoms is quite high, atopic status are not found to be associated with asthma in the salmon workers. None of the examined workers in the study conducted by Bang and co-workers (2005)[2] had specific IgE towards salmon antigen, and only 2,2% of salmon workers displayed IgE towards salmon in the follow up study by Shiryaeva and co-workers (2010)[3].

The upper airways are a portal of entry for workplace-related hazardous material into the respiratory system. Allergens, irritants, and carcinogens can all cause occupational diseases of the airways [4]. A variety of occupational irritant exposures can be associated with airway symptoms, including inhalation of biological agents. Previous research has shown that production workers in the fish industry inhale aerosols containing biological material (bio-areosols) [2, 5]. The biological material may originate from the raw material itself or from microbiological organisms like bacteria and molds in the environment. Inhalation of particulate matter may affect the airway epithelial cells, and surface epithelium in the upper and lower parts of the respiratory tract can contribute to an inflammation response by production and secretion of cytokines.

Occupational reactions to seafood can manifest as rhinitis, conjunctivitis, asthma, urticaria, protein contact dermatitis, and occasionally systemic anaphylactic reactions [6]. Processing of clam, shrimp, and crab is associated with increased risk of occupational asthma, but in a majority of the cases it is not established whether the asthma is of allergic or non-allergic origin [2, 6-11]. Occupational exposure to bony fish (salmon) is also linked to occupational asthma, but the prevalence of diagnosed occupational asthma is lower (2 – 8%) compared to shellfish exposure (4 – 36%) [6, 12, 13].
The observed airway symptoms in fish industry workers may be due to non-allergic inflammatory processes caused by inhalation of biological components in the working environment (endotoxins, moulds, proteases) [2]. Concomitant exposures to agents like histamine, endotoxin and mycotoxins in organic dust and bio aerosols have been known to cause mucous membrane irritation and/or asthma on an inflammatory basis [6, 7, 11]. In the household waste recycling industry, bio-aerosols containing microbial components (endotoxin and β(1-3)-glucans) are shown to induce changes consistent with non-allergic inflammation of both the upper and lower airways, while fungal spores are shown to impact on non-allergic upper airway inflammation [14, 15]. Additionally, there are workplace factors of non-biological origin that also might trigger non-allergic respiratory symptoms of asthma, namely physical factors (hypertonic saline aerosols, cold air, steam vapors) or chemicals (formaldehyde used

in fishmeal production, sulphite preservatives, amines and other anti-microbial agents used to soak gloves, forklift exhaust emissions) [2, 16-18].
As opposed to Tarlo and Liss (2003) reporting more than 90% of occupational asthma cases to be of immunologic origin, Douwes and co-workers (2002) estimate that, based on a systematic review of population based studies, at most, only 50% of asthma cases in the referred populations are attributable to eosinophilic allergic airway inflammation [19, 20]. These latter numbers are also supported by more recent literature [21, 22] and suggests a possible role of neutrophil-mediated asthma triggered by endotoxins and other non-specific irritant factors in causing asthma symptoms [19].

Symptoms occurring from the airways may be indicative of various airway diseases, but in the work related to seafood industry focus is mainly on rhinitis and asthma. **Rhinitis** is defined as inflammation of the nasal mucosa and is characterized by rhinorrhea, sneezing, nasal congestion and/or nasal pruritus [23, 24]. Allergic rhinitis, a subset of rhinitis, is induced by sensitization to allergens with resultant production of allergen-specific IgE. The condition was in 2005 reported to be the most frequent and troublesome allergic disorder in westernized countries [25]. Non-allergic rhinitis (irritant induced) can be secondary to a variety of causes (amongst others inflammation, drug-induced, disease-related) and is mediated by non-IgE-dependent mechanisms. **Asthma** is a lung disease characterized by variable airflow obstruction (which is typically reversible), cellular inflammation, and/or airway hyperresponsiveness. The etiology of asthma may be immunologic where the process is induced by an IgE mechanism or other immune responses such as cell-mediated immunity to specific agents, but it may also be irritant-induced involving non-immunological mechanisms of inflammation. These diseases are termed occupational rhinitis (OR) and occupational asthma (OA) when the initiation and progression of the disease is due to causes and conditions attributable to a particular work environment and not to stimuli encountered outside the workplace [26-29]. Rhinitis and asthma are often associated and share similar immunopathological features, and the documentation of how upper airway disease impacts on the lower airways are increasing [24].

Allergic diseases associated with occupational exposure to crab is quite well characterized [6]; however, earlier researches contain few clear correlations between airway symptoms and enhanced levels of specific immunoglobulins against fish. In most studies regarding occupational exposure to fish the occurrence of symptoms exceeds the occurrence of diagnosed allergic disease.
The fact that the prevalence of work-related airway symptoms is much higher than diagnosed allergic disease due to fish might suggest that not all symptoms experienced by workers can be attributable to fish allergens aerosolized in the working environment. Other allergens such as *Anisakis simplex* (fish parasite) could possibly be important in causing occupational allergic IgE-mediated sensitization and respiratory allergy [30-32], but the observed symptoms could also be caused by non-allergic mechanisms.

Protein-cleaving enzymatic activity by proteases is shown to be important for the induction of cytokine production following exposure to molds [33, 34], mites [35, 36], cockroaches [37], and bacteria [38-41]. The study from Bang and co-workers (2005) has shown that workers in the fish industry might inhale aerosols

containing biologic material that can act as irritants [2]. Additionally, unpublished data from our laboratory show protein-cleaving enzymatic activity in storage water and fish juice sampled from work benches, with the highest activity of trypsin. Protease activity is also found in air samples taken from the breathing zone of the industry workers. Recently, trypsin is shown to be capable of contributing to airway inflammation [42, 43] and asthma [44]. The discovery of the protease-activated receptors (PARs) [45, 46] has lead to an increased understanding of the mechanisms by which trypsin and other proteases may trigger cellular effects in the airways.

Serine proteases

Proteases are traditionally regarded as digestive protein-degrading enzymes but are now gaining recognition as multifunctional hormone-like signalling molecules that are implicated in a vast number of physiological and pathophysiological events [47]. Proteases can regulate cellular signalling events through their interaction with a large variety of targets, including pro-hormones, kininogens, chemokines precursors, proteinase zymogens, and various receptors including the PAR family. Proteases may be divided into five different classes based on their mechanism of catalysis; aspartate, metallo-, cysteine, serine and threonine proteases. The aspartate and metalloproteases use an activated water molecule as a nuclephile to attack peptide bonds, while a catalytic amino acid residue in the active site of the protease serves as the nucleophile for the remaining proteases. Approximately 550 genes encode proteases in the human genome, with the metalloproteases and serine proteases making up the bulk of these [48]. More than one third of all known proteolytic enzymes are serine proteases grouped into 13 clans and 40 families [49]. The family name stems from the nucleophilic serine (Ser) in the enzyme active site, which attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate [50]. Nucleophilicity of the catalytic Ser is typically dependent on a catalytic triad of aspartic acid (Asp), histidine (His), and Ser residues, commonly referred to as the charge relay system [51]. Serine proteases are widely distributed in nature and found in all kingdoms of cellular life as well as many viral genomes. They are usually endopeptidases and catalyze bonds in the middle of a polypeptide chain. However, several families of exoproteases have been described that remove one or more amino acid from the termini of target polypeptide chains. The clan PA proteases bearing the trypsin fold are the largest family of serine proteases and perhaps the best studied group of enzymes. Digestive enzymes such as trypsin cleave polypeptide chains at positively charged (arginine/lysine) residues [52]. A number of key biological processes rely on clan PA proteases and chiefs among them are blood coagulation and the immune response. Besides eliciting a number of physiological processes, endogenous or exogenous proteases in excessive amounts may result in a shift from the normal physiological situation to pathological conditions. The end result of disproportionate protease activity in the airways closely resembles the pathophysiology of rhinitis and asthma and includes the following events listed by Reed (2007) [53];

1) Disruption of tight junctions and desquamation of epithelial cells [34].

2) Chemokine and eotaxin production and release from macrophages and epithelial cells recruit neutrophils and eosinophils [54].
3) Increased bronchial smooth muscle contraction [55, 56].
4) Increased mitogenesis of smooth muscle and fibroblasts, augmented collagen production and promotion of angiogenesis contribute to airway remodelling [57-59].
5) Exogenous proteases from mites and molds augment the IgE response to bystander antigens [60-62]. The intercellular signalling involved in this switch to a Th2 response has not been fully elucidated.

In addition to effects observed in the airways, proteases are found to affect the vascular, renal, gastrointestinal, musculoskeletal, and nervous systems, and can promote cancer metastasis and invasion. Proteases must now be considered as important "hormonal" regulators of inflammation, nociception and cardiovascular function and many physiological responses mediated by serine proteases can occur through the PAR family. An outline of the PAR family and a more detailed description of PAR_2 mediated activation and signalling is given in the below sections.

Protease activated receptor (PAR) family

The protease-activated receptors (PARs) belong to family 1 of the G protein-coupled superfamily of receptors, branching off from relatedness to the neurokinin, substance P, luteinizing hormone- and thyroid-stimulating hormone receptors [63]. G protein-coupled receptors (GPCRs) consist of a single peptide chain that threads back and fourth across the cellular lipid bilayer seven times giving rise to three intracellular and three extracellular loops, an extracellular N-terminus and a C-tail within the cell. Upon activation the intracellular face of the receptor interact with G proteins located at the intracellular side of the plasma membrane [64]. After stimulation and coupling to various G proteins the PARs activate signal transduction pathways resulting in rapid transcription of target genes [65], se figure 1. Activated PARs also interact with various adaptor proteins that facilitate signal transduction independent of heterotrimeric G protein coupling, like β-arrestins and Jab1 (Jun activating binding protein-1) [66].

The hallmark that singles out the PARs from other G protein-coupled receptor systems is the unique proteolytic activation mechanism that reveals a cryptic amino-terminal tethered ligand that folds back and activates the cleaved receptor [67]. Rather than being activated by simple ligand occupancy the PARs are activated enzymatically through proteolysis of the receptor. Specific residues within the enzymatically revealed tethered ligand domain (about six amino acids) are believed to interact with extracellular loop 2 and other domains of the receptor resulting in activation [68], see figure 2a. Thus, PARs are not activated like "classical" receptors because the specific receptor-activating ligand is part of the receptor itself, whereas the circulating agonist is a relatively non-specific proteinase that does not behave like a traditional hormonal regulator [65].

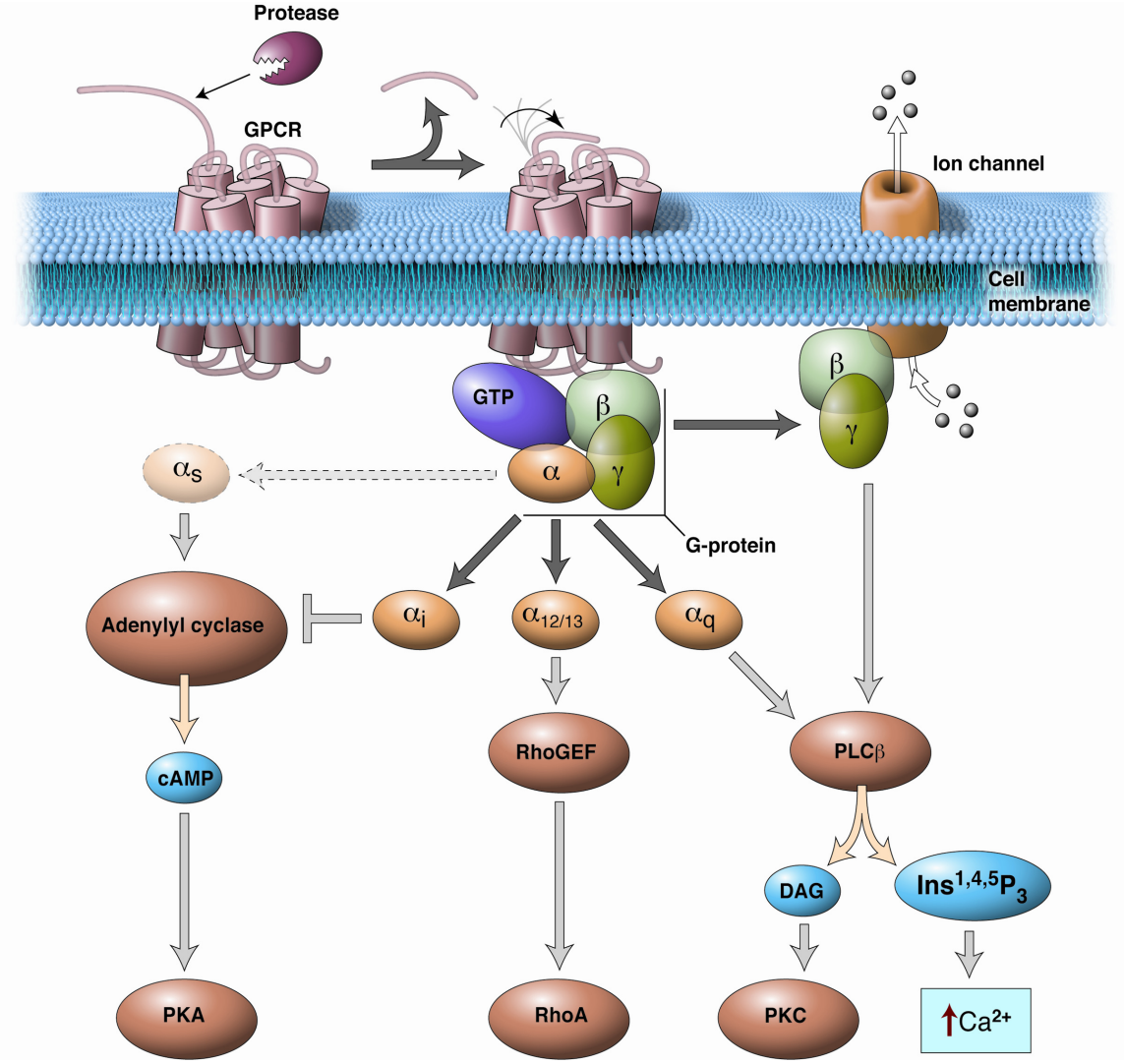

Figure 1

G protein-mediated signalling by GPCRs

In the classical view of G protein-coupled receptor (GPCR) signalling, receptor activation is achieved by agonist binding to extracellular and/or transmembrane regions of the receptor or protease cleavage of the N-terminal end in the case of PARs. The activated GPCR acts as a guanine nucleotide exchange factor, catalysing the exchange of GDP for GTP on the Gα subunit and inducing dissociation of the Gα and Gβγ subunits from each other and from the GPCR. Activated α-GTP subunits, of which there are multiple subtypes, including Gαs, Gαi, Gα12/13 and Gαq, subsequently bind to and regulate the activity of effectors such as adenylyl cyclase, RhoGEF and phospholipase Cβ (PLCβ). These modulate downstream effectors directly or by generating second messengers (such as cyclic AMP, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ($Ins^{1,4,5}P_3$) that modulate further downstream effectors, such as protein kinase A (PKA) and protein kinase C (PKC). Following their liberation from the heterotrimeric G protein complex, the βγ subunits can also bind to and regulate certain downstream effectors, such as ion channels and PLCβ.

Adapted from Ritter and Hall (2009), *Nat Rev Mol Cell Biol* 10 (12): pp 819 – 830 [69].

To date four PARs have been identified by molecular cloning; PAR_1, PAR_2, PAR_3 and PAR_4 [45]; three are originally considered targets for thrombin (PAR_1, PAR_3, and PAR_4) and a single receptor is originally thought to be targeted by trypsin and mast cell tryptase (PAR_2). The proteolytic cleavage of PARs is primarily mediated by a well characterized family of enzymes that require serine within the active site; the serine proteases [45]. Serine proteases are highly active mediators with diverse biological activities. So far, proteases have been considered to act primarily as degradative enzymes in the extracellular space. However, their biological actions in tissues and cells suggest important roles as a part of the body's hormonal communication system during inflammation and immune response [65]. Several proteases have been identified that are capable of activating each receptor and serine proteases may originate endogenously from the coagulation cascade, inflammatory cells, and the digestive tract or exogenously from invading pathogens and particle containing vectors like bio aerosols. Short synthetic peptides (PAR activating peptides; PAR-APs) based on the proteolytically revealed tethered ligand sequences can serve as selective receptor agonists [65], see fig 2b. Some PAR-APs activate more than one PAR, but some (like the PAR_2-AP SLIGRL-NH_2) are highly specific only being able to activate one PAR.

The discovery of the PAR family initiated intensive investigations into the functions of these receptors. The general approach has been to map receptor distribution at the tissue and cellular level, and to examine the biological effects of PAR agonists in different systems. This approach has provided a large body of information that suggests that proteases and their receptors play important roles in tissue responses to injury, including inflammation, pain and healing. The use of genetically modified animals (PAR knockout and transgenic mice) in models of disease further supports an important role of these receptors in disease mechanisms [70].

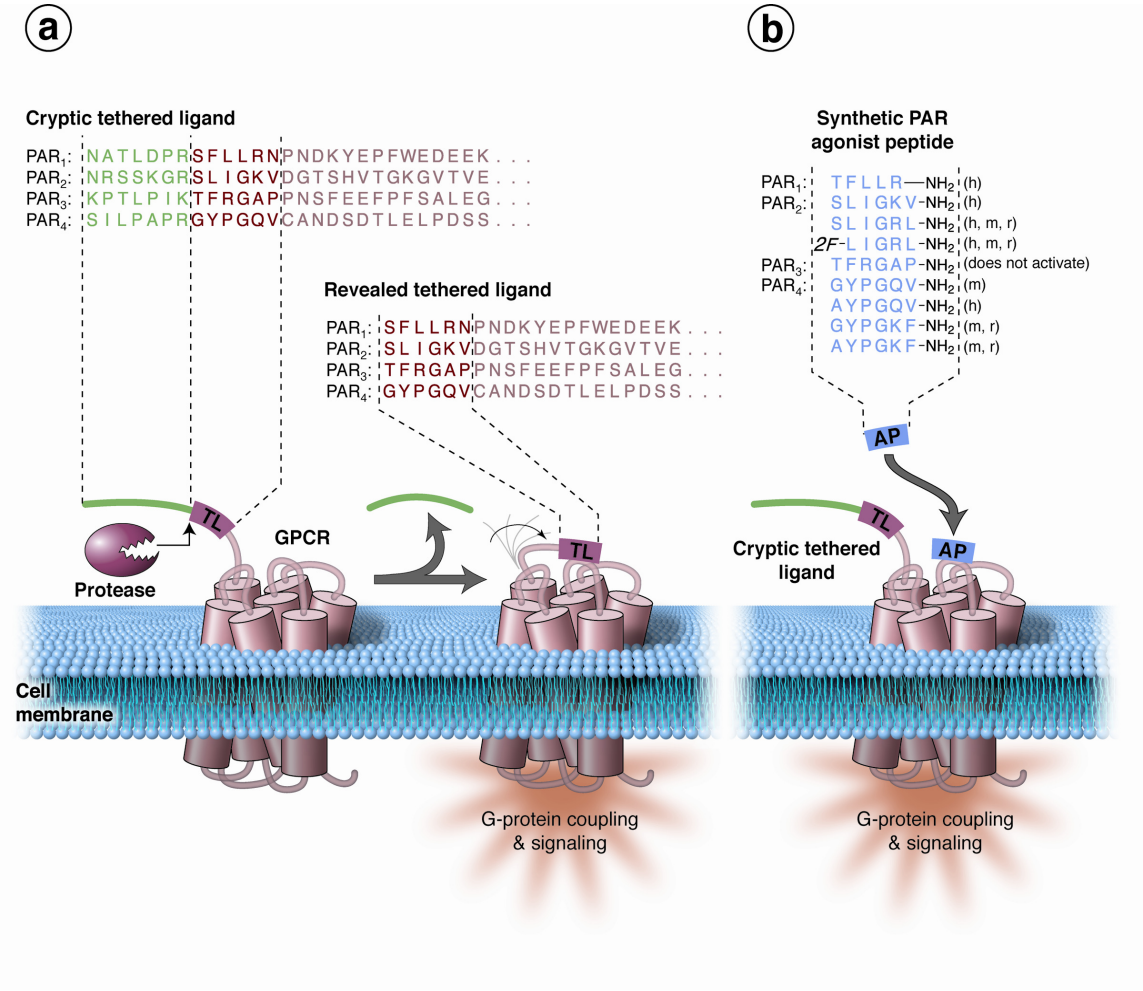

Figure 2

Mechanisms of PAR activation.

(**a**) Activation of PAR signalling by proteinase-mediated cleavage of receptor N-terminus to reveal the tethered ligand (TL). (**b**) Activation of PAR signalling by exogenous application of synthetic PAR agonist peptide (AP) without the need for proteolytic revealing of the tethered ligand.

(GPCR) G protein-coupled receptor, (PAR) proteinase-activated receptor, (h) human, (m) murine, (r) rat.

Adapted from Ramachandran and Hollenberg (2008), *Br J Pharmacol* 153, Suppl 1: pp S263 – S282 [47].

PAR_2

The discovery of the thrombin receptor (PAR_1) in 1991 prompted an intense search for similar receptors for other proteases. The search was fruitless until the serendipitous discovery of a receptor for trypsin by the Sundelin group in 1994. The receptor, now named PAR_2, was identified by screening a mouse genomic library using degenerate primers to the second and sixth transmembrane domains of the bovine neurokinine 2 receptor [46, 71, 72]. A clone was found that encoded a protein of 397 residues with the typical characteristics of a GPCR and with ~ 30% amino acid identity to human PAR_1. Significant heterogeneity was observed in the extra membranous domains, including the C-terminal tail and the N-terminus that is 29 amino acids shorter than in PAR_1 and lacks a hirudin-like thrombin-binding domain [45]. The receptor contained a potential cleavage site within the extracellular amino terminus of 46 residues that was favourable for trypsin. Indeed, trypsin was found to cleave the receptor at this arginine-serine bond to expose the tethered ligand domain responsible for activating the receptor [70].

Mechanisms of activation

As previously described, the general mechanism by which proteases cleave and activate PARs is the same: the proteases cleave at specific sites within the extracellular amino terminus of the receptors; this cleavage exposes a new amino terminus that serves as a tethered ligand domain which binds to conserved regions in the second extracellular loop of the cleaved receptor, resulting in initiation of signal transduction [45, 67, 73]. There is currently no known function of the amino-terminal fragment of the receptor that is removed by proteolysis. Trypsin cleaves PAR_2 at $SKGR^{36} \downarrow S^{37}LIGKV$ to reveal the amino-terminal tethered ligand $S^{37}LIGKV$ in humans and $S^{37}LIGRL$ in rat [46, 71]. Several observations support this mechanism of activation; 1) Mutation of the cleavage site prevents trypsin cleavage and signalling, indicating the importance of this site for activation of PAR_2. 2) Exposure of cells to trypsin results in loss of immunoreactivity to an antibody against an amino-terminal epitope, which indicates that trypsin cleaves intact PAR_2 at the cell surface [74].

Synthetic peptides corresponding to the tethered ligand domain activate PAR_2 without the need for receptor cleavage. Analyses of analogues of the PAR_2 agonist peptide (AP) have identified the residues that are essential for biological activity. In general, the rat/mouse peptide SLIGRL is slightly more potent that the human agonist SLIGKV in activating both human and murine PAR_2 [75]. Only the first five amino acids of the PAR-activating peptides are critical and a short (four or five peptide residues) peptide motif appears to be sufficient for receptor activation [76]. Analysis by alanine scanning indicates that Leu^2 and Arg^5 are essential for activity. Substituting the positive Arg^5 with either an amino acid with a neutral side chain (alanine) or a negative side chain (glutamic acid) in the peptides SLIG**A**L or SLIG**E**L reduces their potency for activating PAR_2 by at least 32-fold (SLIGAL) and 130-fold (SLIGEL) measured by their ability to cause a rise in intracellular calcium signalling in transfected KNRK cells expressing wild type ratPAR_2 constructs [77]. In addition to SLIGRL/SLIGKV there are

several other peptides that may activate PAR_2 and their structure-activity relationship has been extensively studied in bioassay systems including tissue preparations (rat aorta relaxation, rat gastric longitudinal muscle contraction) and cell culture (human embryonic kidney 293 (HEK293) cell calcium signal) [76-78]. The incorporation of an amide at the carboxy terminus of the receptor activating peptides increases peptide potency (SLIGRL-NH_2 > SLIGRL) [76, 79] as well as incorporating an N-terminal furoyl-modifiaction to protect the peptide from endogenous aminopeptidases (2-furoyl-LIGRLO-NH_2 >>SLIGRL-NH_2) [80, 81]. The relative potencies of the PAR_2 selective agonist peptides in either a calcium signalling assay or aorta relaxation assay were found to be: 2-furoyl-LIGRLO-NH_2 >> SLIGRL-NH_2 ≈ trans-cinnamoyl-LIGRLO-NH_2 > SLIGKV-NH_2 > SLIGKT-NH_2 [82].
The PAR_2 agonist peptides are selective for PAR_2, but the fact that they are agonists only at relatively high peptide concentrations, unspecific effects not related to activation of PAR_2 may occur. Because of this, the search for agonists displaying higher potencies is continuous. Recently, two small-molecule PAR_2 agonists have been reported; AC-55541 (N-[[1-(3-bromo-phenyl)-eth-(E)-ylidene-hydrazinocarbonyl]-(4-oxo-3,4-dihydro-phthalazin-1-yl)-methyl]-benzamide) and AC-264613 (2-oxo-4-phenylpyrrolidine-3-carboxylic acid [1-(3-bromo-phenyl)-(E/Z)-ethylidene]-hydrazide) [83]. They each represent a distinct chemical series and show similar potency as 2-furoyl-LIGRLO-NH_2, the most potent PAR_2 agonist peptide so far.

Identification of the docking interactions between tethered ligand domain and receptor is critical for understanding transmembrane signalling by these receptors. Along with studies investigating agonist peptide potencies, analyses of mutant and chimeric receptors and of analogs of APs have identified critical residues of the tethered ligand domains that interact with binding domains of the PARs (extracellular loop II) and which are thus essential for signal transduction. Interactions between the tethered ligand of PAR_2 and the cleaved receptor have been examined by studying chimeras of PAR_1 and PAR_2 and shows that the primary determinant of agonist specificity is extracellular loop 2 (ECL2) [84]. Receptor chimera studies of the human-Xenopus PAR_1 receptor suggest a possible interaction between Arg^5 in SFLL**R**N and Glu^{260} in the second extracellular loop of the PAR_1 [85, 86]. These amino acids are also conserved in PAR_2. Thus, an Arg^5-Glu^{260} interaction might also operate in recognition of the PAR_2 agonist peptide SLIG**R**L by PAR_2.
The important role of residues in ECL2 of PAR_2 for interaction with tethered ligand peptides has also been revealed by the study of mutant receptors and analogs of the PAR_2 agonist peptides [87]. An acidic region (PEE) that is just distal to a highly conserved domain (CHDVL) makes an important contribution to determining the selectivity of PAR_2 agonists, but seems not to have as marked an effect on receptor activation by the trypsin-revealed tethered ligand as for activation by free peptides. However, the different specificities that is shown for PAR_1 and PAR_2 (the PAR_1 agonist peptide SFLLRN is able to activate PAR_2 as well as PAR_1, while the PAR_2 agonist peptide SLIGRL only activates PAR_2) suggests that other direct or indirect interactions between agonists and ECL2 must be responsible for distinguishing the PAR_1 and PAR_2 receptor agonists. In addition to the interaction between the tethered ligand at the amino terminal end (or agonist peptide) and ECL2, other extracellular domains of PAR2 like ECL3 and the amino terminal end may be important in agonist recognition and research indicates that they must interact, directly or indirectly, for proper receptor function [84].

Research has also shown differential activation of PAR_2 by the tethered ligand and soluble peptide analogs. Rat PAR_2 receptor mutants in which the positive arginine at position 41 of the revealed tethered ligand (corresponding to Arg^5 in the soluble peptide analogue SLIGRL) is changed to either a neutral alanine or a negative glutamatic acid will correspond to the synthetic receptor analogues SLIGAL and SLIGEL when revealed by trypsin cleavage. Compared to the wild type receptor these mutated receptors give equivalent responses in intracellular calcium signalling when activated by trypsin. Bearing in mind the fact that the soluble peptide analogues SLIGAL and SLIGEL had marked potency differences compared to SLIGRL, it can be concluded that for PAR_2 activation, the trypsin-revealed PAR_2 tethered ligand sequence interacts differently for receptor activation that does the same peptide sequence as a free peptide [77].

In addition to Arg^5 in the agonist peptide, the first two amino acids of the trypsin-revealed tethered ligand in ratPAR_2 ($Ser^{37}Leu^{38}$) were found to be important in an intracellular Ca^{2+} signalling assay [88]. Here the revealed rat PAR_2 mutated TL sequence SL**AAA**-, was able to stimulate increase in intracellular Ca^{2+}, whereas the mutated TL sequences **LS**IGRL- and **AA**IGRL- were not. In addition, the work showed that whereas the sequence SL**AAA**- could activate calcium signalling as a tethered ligand, the corresponding soluble peptide SL**AAA**-NH_2 was unable to do so. These data points to differences in signal trafficking by PAR_2 depending on whether it is activated by its own proteolytically revealed TL or by an analogous synthetic peptide.

The Hollenberg group has also shown that even though the ability of the revealed tethered ligand to stimulate an elevation in intracellular calcium is lost when the $S^{37}L^{38}$-sequence is mutated to alanines or switched in sequence to $L^{37}S^{38}$, the activation of MAP kinase is retained. This biased signalling with a triggering of MAP kinase but not calcium is also observed for the synthetic PAR-activating peptide SLAAA-NH_2, and its ability to interact with the receptor seems to differ from that of the same sequence when presented to the receptor as a trypsin-revealed tethered ligand, which activates both calcium and MAP kinase signalling [89]. For many other GPCRs, such as those for angiotensin II, dopamine, serotonin, and adrenergic ligands, it is now accepted that there can be differential signalling, depending on the activating ligand. This agonist-dependent differential signalling has been termed “agonist-biased signalling” or “functional selectivity” [90-93].

Although PAR_2 originally is considered to be activated by trypsin, other proteases are capable of cleaving the PAR_2 N-terminal tail under experimental conditions. However, posttranslational modifications of the receptor may influence on the ability of different proteases to cleave and activate PAR_2. PAR_2 is shown to contain at least two sites for N-linked glycosylation: Asn^{30}; 6 residues proximal to the cleavage and activation site, and Asn^{222} in ECL2. The potency by which tryptase (but not trypsin) activates PAR_2 is dramatically increased by mutation in one of the glycosylation sites (Asn^{30}), by enzymatic deglycosylation, or by expression of PAR_2 in glycosylation-defective cells [94, 95]. Thus, glycosylation of the receptor at a site close to the activation site markedly impairs the capacity of tryptase to signal. The reason for this finding is currently unknown, but glycosylation could impede access of the amino terminus of PAR_2 to the active site of tryptase. An important aspect is to

determine if the receptor is similarly glycosylated in tissues and to know if there are mechanisms that deglycosylate the receptor.

Multiple proteases activate PAR_2

The capacity of a protease to signal in intact tissues depends on many factors [73];
First, the protease(s) must be generated or released in sufficient concentrations to activate PAR_2. Although the catalytic properties of proteases would ensure that even very low concentrations could eventually cleave all receptors on the surface of the cell, it is the rate of hydrolysis of PARs that determines the magnitude of the resulting cellular signal [96]. Second, efficient hydrolysis and activation of PAR_2 may require the presence of accessory co-factors, for example tissue factor (TF) in the case of FVIIa-FXa receptor activation. Finally, the capacity of a protease to signal will depend on the availability of protease-inhibitors that serve to dampen the effects of many proteases in vivo. Although trypsin is a very potent activator of PAR_2 in cultured cells, trypsin inhibitors are widely expressed and may well limit the capacity of trypsin to signal in intact tissues. Remembering these caveats, many proteases have been identified that are capable of activating PAR_2 in different cell lines under experimental conditions, but the endogenous activator of PAR_2 is uncertain in most situations [73].

1) Pancreatic and extrapancreatic trypsins

There are at least three distinct trypsin genes in humans: trypsin I (cationic trypsin), trypsin II (anionic trypsin), and mesotrypsin. **Trypsin I** [71, 97], **trypsin II** [98] and **trypsin IV** (a splice variant of mesotrypsin) [99] are all found to be capable of activating PAR_2. Trypsins are widely distributed enzymes that are expressed by pancreatic as well as extrapancreatic cells including endothelial cells [100], epithelial cells, leukocytes, and cells in the nervous system [101] and in tumors [102, 103].
The potential of trypsins to signal to cells by cleaving PAR_2 depends on the release of the zymogen trypsinogen, the presence of enteropeptidase (which activates trypsinogen), and the existence of the large array of endogenous trypsin inhibitors. Luminal trypsin (trypsin I and II) in the small intestine reach a concentration of 1 µM in feeding rat, a concentration which is more than capable of strongly activating PAR_2 at the apical surface of enterocytes (EC_{50} ~ 5 nM) [104]. Trypsin II isolated from conditioned medium from colon cancer cell lines can cleave and activate PAR_2, and since these cells express PAR_2 it is theoretically possible that trypsin II could regulate cells in an autocrine manner [105]. Pancreatic trypsinogens are also prematurely activated in the inflamed pancreas where they are released into the interstitial fluid and vasculature and could activate PAR_2 in pancreatic acini, duct cells, and nerves [106]. Trypsinogen IV is invariably co expressed with PAR_2 in epithelial cell lines, endothelial cell lines, and human colonic mucosa and trypsin IV cleaves and activates PAR_2 [73]. Of particular interest is that trypsin IV is resistant to most proteinaceous trypsin inhibitors that effectively inhibit trypsins I and II [107]. In the airways trypsinogen is found to co-localize with PAR_2 in Clara cells of the epithelium and to be expressed by cells just above the basal layer of human bronchioles [108, 109]. Trypsin II is found to co-localize with PAR_2 in bronchoalveolar epithelium from preterm infants with acute and chronic lung injury [110]. Human airway trypsin-like protease (HAT) is a monomeric serine

protease that is found specifically in ciliated epithelial cells of the airways [111, 112]. HAT is shown to activate PAR_2 in primary human bronchial epithelial cells and human bronchial fibroblasts [113, 114]. The results from Cocks [108, 109] and Cederqvist [110] strongly suggest that a trypsin-like substance is present in the airway epithelium; however, in immunohistochemistry cross-reactions may occur and raises uncertainty whether the trypsin immunoreactivity is caused by trypsin itself or other trypsin-like substances like HAT.

2) Mast cell tryptase

There has been considerable interest in mast cell tryptase (MCT) as an activator of PAR_2. Tryptase is the most abundant protease of human mast cells – it comprises up to 25% of the total cellular proteins and is expressed by almost all subsets of human mast cells [115]. Human mast cells express at lest five distinct tryptase genes: α, βI, βII, βIII and transmembrane tryptase, and splice variants also exist. Mast cell tryptase purified from human lung is shown to cleave a peptide based on the PAR_2 cleavage site, suggesting that this protease is a potential physical activator of PAR_2 [116]. Trian and co-workers (2006) have shown by RNA interference that tryptase activates PAR_2 in human airway smooth muscle cells [117]. Many of the proinflammatory and mitogenic effects of tryptase are mimicked by PAR_2-APs and absent in cell types not expressing PAR_2. This suggests that tryptase exerts its effects through PAR_2 in various cell types, though the molecular form of tryptase responsible for the observed PAR_2 effects in each case is largely unknown [57, 118-123]. However, tryptase is considerably less potent than trypsin. Because tryptase is a large and poorly diffusible protease (a 134 kDa tetrameric protease in the form of a flat ring of four monomers), it is likely that tryptase signals in a paracrine manner to cells that are in close proximity to mast cells, such as sensory nerves that express PAR_2, which participate in inflammation and pain [124-126]. There is evidence for the fact that tryptase can activate PAR_2 *in vivo* under conditions of inflammation and mast cell activation when large amounts of tryptase are released close to PAR_2 expressing cells. Thus, injected tryptase has proinflammatory and hyperalgesic actions in mice that are not observed in PAR_2-deficient animals [126, 127].

3) Cell-surface proteases

Certain anchoring proteins may serve as co-factors that facilitate the capacity of certain proteases to activate PARs, but proteases that are themselves integral membrane proteins can also activate PAR_2. Membrane-type serine protease 1 (MT-SP1) is a type II integral membrane protein with an extracellular protease domain [128]. Analysis of the substrate specificity of MT-SP1 suggests PAR_2 as a potential substrate, and both MT-SP1 and PAR_2 are co-expressed at the surface of certain cell types (e.g. PC-3 cells). It remain to be determined if the membrane-bound MT-SP1 can activate PAR_2 under more physiological circumstances.

4) Coagulation factors

Serine proteases from the coagulation cascade are perhaps the best characterized activators of PARs as thrombin has been extensively studied as an intrinsic agonist that activate PAR_1, PAR_3, and PAR_4 at the surface of platelets resulting in aggregation, a process which contributes to hemostasis [129].

Coagulation factors VIIa (FVIIa) and Xa (FXa) are proteases that act upstream of thrombin in the coagulation cascade and require co-factors to interact with their substrates. These proteases elicit cellular responses, but their receptor(s) have not been identified. The tissue factor (TF)-FVIIa-FXa complex may signal by cleaving PAR_2 on epithelial (keratinocytes) and endothelial cells, the latter of particularly importance in inflammation [130, 131]. This activation is dependent on the presence of accessory proteins and coagulation factor VIIa (FVIIa) activates PAR_2 only in cells that also express tissue factor (TF), an integral membrane protein which binds and concentrates factor VIIa at the cell surface in the vincity of PAR_2 [130]. The FVIIa/TF complex also generates factor Xa (FXa) from FX at the cell surface, which facilitate PAR_2 activation.

5) Tumor-derived proteases: kallikreins

The family of kallikreins (KLKs) has attracted substantial attention as a group of serine proteases linked to cancer-associated pathophysiology [132, 133]. The human KLKs, which are secreted as inactive zymogens, can exhibit either trypsin (twelve family members) or chymotrypsin-like (three family members) activity upon proteolytic activation [133]. Although the KLKs are differentially expressed in cancer and other diseases in relation to normal tissues [132-134], little information exist about the functions that these enzymes might play *in vivo* [135]. Recent work by Oikonomopoulou and co-workers (2006a,b) hypothesize that the tissue kallikreins can signal to cells by cleavage and activation of PARs [136, 137]. KLK5, 6 and 14 are all found to cleave synthetic peptides representing the cleavage-activation sequences of PAR_2 and cause increase in intracellular Ca^{2+} in target cells. There are distinct differences between the three tested kallikreins in terms of their selective actions on each of the PARs. KLK14 activate both PAR_2 and PAR_4, and may activate or inactivate PAR_1, while KLK5 and 6 preferentially activate PAR_2. Like trypsin, all three KLKs can activate vascular endothelial PAR_2 (rat aorta) to cause NO-dependent relaxation [136]. Further work is required to evaluate the potential inflammatory and nociceptive roles that KLKs may play. Because of their ability to regulate tissues via PARs, their wide expression in regions where trypsins may not be produced and their possible participation in enzymatic cascades, the KLKs represent important potential physiological regulators of tissue function *in vivo* [135].

6) Other mammalian enzymes

A tryptic-like serine protease purified from rat brain, P22, degrades matrix and can signal to cells by activating PAR_2 [138]. Acrosin, a trypsin-like serine protease found in large quantities in the acrosomal body of spermatozoa of all mammals activate PAR_2 in stably transfected Chinese hamster ovary cells [139]. Furthermore, airway epithelium expresses acidic mammalian chitinase [140] which may be a possible activator of PAR_2 as chitinase from *Streptomyces griseus* is shown to activate PAR_2 in human airway epithelial cells [141]. Mammalian chitinase is thought to be a mediator of disease manifestation in an experimental model of asthma.

7) Non-mammalian proteases

A number of non-mammalian proteases from mites, bacteria, mold/fungi, cockroaches and fish have been found to signal to mammalian cells by cleaving PARs [1, 38-41, 141-150]. Although many of the exogenous proteases that activate PAR_2 are serine proteases, other proteases like the cysteine- and metalloproteases are also found to be capable of activating PAR_2.

The dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* produce a series of proteinases that are allergens in the airway epithelium. Der P3 and Der P9 are serine proteases that can cleave fragments of PAR_2 at the activation site, and desensitization experiments suggest that the effects of these proteases on airway epithelial cells are mediated in part by PAR_2 [149]. The bacteria *Porphyromonas gingivalis* is a major mediator of periodontitis in humans, and bacterial arginine-specific gingipains-R (RgpB, a cysteine protease) can activate PAR_2 transfected cells in an oral epithelial cell line to induce release of the powerful proinflammatory cytokine IL-6 [40]. Thermolysin [150], serralysin from *Serratia marcescens* [38] (both metalloproteases) and LepA from *Pseudomonas aeruginosa* (large exoprotease with a trypsin-like serine protease motif) [39] are additional bacterial proteases that are found to activate PAR_2 in experimental settings including HBE (human bronchial epithelial cells) and EBC-I cells (human lung squamous cell carcinoma). Chitinase (a hydrolase) from *Streptomyces griseus* is a potent proteolytic activator of PAR_2 and directly induce PLC/IP3-dependent Ca^{2+} signalling in human airway epithelial cells [141]. Additionally, extracts from *Aggregatibacter actinomycetemcomitans* are shown to induce secretion of IL-8 and expression of ICAM-1 via PAR_2 activation in gingival epithelial cells [41].

The mold allergen and serine protease, Pen c 13 is the immunodominant allergen produced by *Penicillium citrinum* and induces IL-8 expression in human airway epithelial cells (A549) by activating PAR_2 (and PAR_1) [142].

PAR_1 and PAR_2 contribute to inflammation and immunity to the fungi *Candida albicans* and *Aspergillus fumigatus*. Activation of Toll-like receptors (TLRs) by fungi has unmasked an essential and divergent role for PAR_1 and PAR_2 in downstream signalling and inflammation. TLRs activate PARs and triggers distinct signal transduction pathways. Inflammation was promoted by PAR_2 activation in response to *Candida* and by PAR_2 inhibition in response to *Aspergillus* [146].

Serine proteases from cockroach activate PAR_2 in human bronchial epithelial cells [147, 148], human keratinocytes [143], and mouse lung fibroblasts [144] and regulate IL-8 expression via nuclear factor for interleukin-6 (NF-IL6) and extracellular-signal regulated kinase (ERK) in human bronchial epithelial cells. PAR_2 activation by cockroach allergens is also shown to delay epidermal permeability barrier recovery in murine skin [143].

Purified salmon trypsin is shown to induce secretion of IL-8 from cultured airway epithelial cells (A549), an effect mediated by activation of PAR_2 as knockdown of PAR_2 by the use of siRNA almost completely abolishes the dose-dependent IL-8 release. Purified salmon trypsin is also found to promote DNA-binding of the transcription factor NF-κB via activation of PAR_2. These findings suggest that purified salmon trypsin behaves similarly to mammalian trypsin regarding the ability to activate PAR_2, but closer comparisons have not been made [1]. In addition, trypsins from sardine and king crab are shown to cleave and activate PAR_2. Both sardine and king crab trypsin induces the secretion of IL-

8 from human airway epithelial cells (A549), an effect at least partly mediated by PAR_2, but displays individual differences in transformation of the NF-κB signal [145].

Proteolytic inactivation of PAR_2

Some proteases are also shown to be capable of inactivating PAR_2 by cleaving the N-terminal of the receptor in a way that does not produce an activating tethered ligand sequence, leaving the receptor refractory to subsequent activation by other proteases. Additionally, proteases that cleave the binding domain in extracellular loop II would generate unresponsive receptors.
Neutrophil elastase (NE), cathepsin G (CG), and proteinase 3 (PR3) are serine proteases stored in the azurophilic granules of polymorphnucleated neutrophils (PMN). They participate in destruction of invading microorganisms, but can also proteolyze endogen components like extracellular matrix and immune effector proteins when released in excessive amounts. NE and CG are shown to disarm PAR_2 most likely by cleavage at sites downstream of the activation site in human respiratory epithelial cells (16HBE, A549) [151, 152]. Although not tested in cell lines, PR3 is shown to cleave the extracellular N-terminal fragment of PAR_2 at disabling sites downstream from Ser^{37} like NE and CG [152].
In addition to the endogen proteases originating from neutrophils, elastase from *Pseudomoas aeruginosa* (EPa, an elastolytic metalloprotease also termed LasB or pseudolysin) is shown to disable PAR_2 in respiratory epithelial cells [153]. EPa cleaves the N-terminal domain of PAR_2 from the cell surface without triggering receptor endocytosis as trypsin does. This cleavage does not activate PAR_2 (evaluated by measurement of cytosolic calcium, PGE_2, and IL-8 production), but rather disarms the receptor for subsequent activation by trypsin, but not by the synthetic receptor-activating peptide, SLIGKV-NH_2. Proteolysis by EPa of synthetic peptides representing the N-terminal cleavage/activation sequences of either human or rat PAR_2 indicates that cleavages resulting from EPa activity (at Ser^{37} – Leu^{38} and Gly^{52} – Val^{53}) would not produce receptor-activating tethered ligands, but would disarm PAR_2 in regard to any further activating proteolysis by activating proteases. Thermolysin is also shown to render PAR_2 refractory to trypsin but not agonist peptide stimulation in A549, suggesting that this bacterial metalloprotease might disable PAR_2 in this cell type compared to the human bronchial epithelial cell line where it is shown to activate the receptor [150].

Some proteases can cleave PARs at several sites, including activation and disabling sites, and the net result depends on the efficiency of cleavage at different locations. Tryptase can cleave PAR_2 both at the activation site (Arg^{36} – Ser^{37}) and at the Lys^{41} – Val^{42} site which could inactivate the receptor [122]. In this case, the activating cleavage is more important since tryptase is found to be a PAR_2 activator. This dual cleavage is presumably a concentration-dependent effect as tryptase at 1 nM cleaves the peptide at the PAR_2 activation site, while cleavage at the inactivation site is detected first at 100 nM.

Receptor interactions

PARs are frequently co-expressed and interactions between receptors in the same cell can have important functional consequences [73]. GPCRs can form homodimers and heterodimers with important consequences for signal transduction. Although the principal mechanism of PAR activation is **intra**molecular (the unmasked tethered ligand binds to the cleaved receptor), there are several examples of **inter**molecular interactions between different PAR molecules. Intermolecular signalling, by which a cleaved receptor can activate an uncleaved receptor, was first demonstrated for PAR_1 [154]. There is also evidence of intermolecular signalling between different PARs and peptides corresponding to the tethered ligand of PAR_1 (SFLLRN) are shown to activate PAR_2, but not vice versa [75]. Research has also shown that cleaved PAR_1 receptor is able to interact with and signal through uncleaved PAR_2 [155]. This novel form of intermolecular signalling between different PARs clearly requires the close association of receptors at the cell surface, which could be influenced by levels of expression or by anchoring proteins that may affect mobility of receptors in the membrane [73].

PAR_2 is also shown to interact with other receptors besides its family members. In mouse and man, the TLRs represent a family of > 10 single-transmembrane classical pattern-recognition receptors (PRRs) that detect chemically conserved microbial components, for example, lipopolysaccharide (LPS), lipopeptides, and RNA [156-158]. Innate immunity has largely been attributed to the capacity of PRRs to respond to conserved pathogen-associated molecular patterns (PAMPs), whereas PARs have generally been considered to be “sensors” of the extracellular proteolytic environment.
In transiently transfected HEK293 cells and a non-transfected colonic epithelial cell line (SW620), PAR_2 is found to interact with Toll-like receptor 4 (TLR4) showing a synergistically response to PAR_2 agonist peptide and LPS [159]. PAR_2 and TLR4 are believed to form a heterophilic receptor interaction that centers on shared utilization of TLR adapter proteins, leading to intracellular signalling (NF-κB activation) upon PAR_2 engagement with agonist peptides. The synergistically NF-κB activation is found to be MyD88-dependent, one of two major signalling pathways activated by TLR4.
PAR_2 activation is also found to deliver intracellular signals that intersect with TLR/IL-1R (interleukin-1 receptor) signalling pathways [159-162]. Cooperation between PAR_2 and TLR2, TLR3, or TLR4 for NF-κB-dependent IL-8 mRNA induction is observed in the mucosal epithelial cell lines A549 (human airway epithelial cells) and SW620 (colon epithelial cells). PAR_2 is also shown to execute negative regulation of the TLR3 antiviral pathway [163]. This collaboration between PAR_2 and TLRs imply a previously under-appreciated role of PARs as an additional level of the innate immune defence, apart from the classical pattern-recognition receptors.
Furthermore, PAR_2 is shown to transactivate epidermal growth factor receptor (EGF-R) in a human colon cancer cell line and intestinal epithelial cells stimulated with both trypsin and PAR_2 agonist peptide, resulting in cellular proliferation through ERK 1/2 activity [164, 165]. These results indicate that PAR_2 may play a role in the control of tissue growth through promotion of cellular proliferation in colon cancer. Additionally, the oncogene variant of EGF-R is associated with increased expression of PAR_2, yielding tumour cells that are hypersensitive to contact with blood borne proteases and contribute to formation of a growth promoting microenvironment [166].

Signal transduction

Although the knowledge of PAR_2 signalling pathways is more limited than the existing information on PAR_1 mediated signalling events, ongoing research constantly reveal new information regarding PAR signalling. With respect to the coupling of PAR_2 to the various G proteins, the existing information does not originate from airway cells. However, when describing the signal transduction cascades downstream of G proteins, I will focus on current knowledge about PAR_2 mediated signalling in airway cells. Still, it has to be kept in mind that the various signalling events following receptor activation may differ between different cell lines/types even though they all originate from the airways. For more information about PAR_2 signalling events in other cell types/tissues the readers are referred to reviews from MacFarlane *et al* 2001 [45], Coelho *et al* 2003 [70], Ossovskaya and Bunnett 2004 [73], Steinhoff *et al* 2005 [65], and Ramachandran and Hollenberg 2008 [47].

Signal transduction following receptor activation commences with coupling of PARs to heterotrimeric G proteins at the plasma membrane, often called transducers of the signal. The activated G protein subunits (α and βγ) can then associate with downstream effectors to modulate various aspects of cellular physiology. PAR_1 is known to couple with multiple G proteins, including $G_{\alpha q}$, $G_{\alpha i}$, and $G_{\alpha 12/13}$ families [45, 65], but our knowledge of PAR_2 interactions with G proteins and subsequent intracellular signalling has been studied in less detail. It is likely that PAR_2 couples to $G_{\alpha q}$ since PAR_2 activators stimulate the generation of inositol-tris-phosphate (IP_3) along with diacyl-glycerol (DAG) and subsequent intracellular Ca^{2+} mobilization in PAR_2 transfected cells and several cell types including enterocytes, kerationocytes, myocytes, neurons, astrocytes and tumor cells [46, 71, 97, 104, 119, 120, 167, 168]. Pertussis toxin-sensitive Ca^{2+} signalling has been demonstrated in Xenopus oocytes in response to trypsin indicating a potential involvement of $G_{\alpha i}$ [45, 169]. However, trypsin stimulation rendered PAR_2 signalling unaffected by pertussis toxin in PAR_2 transfected cells and enterocytes, suggesting that it does not signal through $G_{\alpha i}$ in these cells [170]. McCoy and co-workers (2010) have shown that in contrast to PAR_1, all of the $G_{\alpha i}$ subunits binds only weakly or not at all to PAR_2 in transfected COS-7 cells and wild type Neu7 astrocytes [171]. The same study reports the formation of a stable complex with PAR_2 and $G_{\alpha 12/13}$ that leads to Rho A activation following agonist peptide stimulation.
The coupling of PAR_2 to G proteins may very well differ in different cell types and tissues, and under different conditions. Additionally, one agonist stimulating PAR_2 may lead to activation of different signalling pathways (eks intracellular Ca^{2+} signalling and MAP kinase activation) that depends upon coupling of distinct G proteins to the activated receptor. The generation of intracellular Ca^{2+} signalling following trypsin stimulation in $rPAR_2$ transfected KNRK cells is $G_{\alpha q}$-mediated, whereas MAP kinase activation in the same cell type following trypsin stimulation is only partially $G_{\alpha q}$-dependent but seems to be dependent on $G_{\alpha 12/13}$ coupling and Rho activation [89].

Interestingly, PAR_2 has been shown to be able to signal via an arrestin-mediated process that can be independent of G protein interactions in mouse embryonic fibroblasts and a human breast cancer cell

line [172]. This G protein independent signalling might involve PAR heterodimers (for example PAR_2/PAR_1) and may explain the "dual" actions that PAR_2 displays in certain settings, triggering either inflammatory or anti-inflammatory responses. Furthermore, PAR_2 is shown to promote the formation of a complex containing β-arrestins, cofilin and chronophin (the upstreams activator of cofilin) in primary leukocytes and cultured cells (mouse embryonic fibroblasts and human breast cancer cells), a complex that is important in leukocyte chemotactic migration by directing localized actin filament severing and membrane protrusions [173]. In addition to the β-arrestins, PAR_2 has been reported to bind to the Jun activating binding protein-1 (Jab1), a protein that stabilizes complexes of the AP-1 family members c-Jun and Jun D with their DNA-binding sites [174]. The binding of Jab1 and PAR_2 induce activation of the transcription factor c-Jun and subsequent gene transcription.

Following activation of the G proteins, primary effectors generate secondary messenger molecules like IP_3, DAG and Ca^{2+} creating signals that can diffuse within the cell by activating secondary effectors and other downstream signalling components. PAR_2 activation is shown to turn on the primary and secondary effectors phospholipase C [175] and protein kinase C [176] in human airway smooth muscle and human airway epithelial cells (A549) respectively. Active phospholipase C might be due to PAR_2 activation in human airway epithelial cells (A549) stimulated with the mold allergen Pen c 13 [142]. Recently, it has been revealed that regions within the PAR_2 C-terminal tail are critical for phospholipase C-mediated IP_3 formation and Ca^{2+} signalling as opposed to the activation of MAP kinase that is triggered by other intracellular PAR_2 sequences [177].
The MAPK cascade consists of various pathways involving multiple kinases and ERK 1/2 activation has been connected to PAR_2 in the airways by stimulating human bronchial epithelial cells (16HBE14o) [147] and human airway epithelial cells (A549) [176, 178, 179] with selective PAR_2 peptides. Human airway trypsin-like protease (HAT) is found to stimulate primary human bronchial fibroblasts through a PAR_2-dependent MEK/ERK1/2 mediated pathway [113]. The activation of ERK 1/2 signalling is facilitated by PAR_2 binding to and co-internalizing with β-arrestins in endocytic vesicles, an event that mediates changes in the actin cytoskeleton and cell migration [170, 180] as well as contributing to the formation and secretion of IL-8 and PGE_2. In addition to ERK 1/2, PAR_2 is shown to trigger phosphorylation of p38 and JNK in human airway epithelial cells (A549) [176, 178, 179, 181]. Furthermore, PAR_2 activation is shown to up-regulate phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX-2) in A549 cells, an effect shown to trigger the formation of PGE_2 [176]. The PAR_2 triggered PGE_2 formation as well as IL-8 secretion in A549 cells is also shown to involve the Src family tyrosine kinase and the epidermal growth factor receptor tyrosine kinase (EGFR-TK) [176, 181], mediators that links PAR_2 to the transactivation of EGFR.
The activation of intracellular signalling pathways leads to the generation of various products, both molecules that acts intracellularly and secreted molecules with autocrine, paracrine and endocrine effects. The production of these effectors involves gene transcription, a process initiated and controlled by transcription factors. PAR_2 mediated activation and DNA-binding of NF-κB has been demonstrated in human airway epithelial cells (A549) [1, 178, 182]. The PI3K/Akt pathway is capable of activating I-κB kinase α (IKKα), followed by phosphorylation of I-κB and activation of NF-κB in TNF

stimulated cells (transfected HEK293) [183]. Moriyuki and co-workers (2009) have shown that the PI3K/Akt pathway is involved in the PAR_2-triggered NF-κB signalling in human airway epithelial cells (A549), and that the PAR_2 triggered phosphorylation of Akt is partially suppressed by MEK-inhibition [184]. Apart from NF-κB, CREB (cAMP responsive element binding protein) has been shown to be downstream of ERK 1/2 and mediate up-regulation of COX-2 through the β-catenin/T cell factor transcription pathway following PAR_2 activation in A549 cells [185]. This PAR_2 up-regulated COX-2 expression reveal new cellular mechanisms by which serine proteases may participate in chronic mucosal inflammation and associated conditions. Phosphorylation of CREB is also shown to be involved in PAR_2 induced PGE_2 release from A549 cells through up-regulation of COX-2 [184].
In addition to NF-κB, activation of PAR_2 is shown to facilitate DNA-binding of the transcription factor AP-1 in human airway epithelial cells (A549) [178] and of NF-IL6 in human bronchial epithelial cells (16HBE14o) [148]. The binding of NF-κB is shown to be essential for the induction of IL-8 gene transcription, while these two latter transcription factors (AP-1 and NF-IL6) are shown to be essential for maximal production of IL-8 [186-189].
Besides signal transduction leading to the release of cellular mediators, PAR_2 activation is reported to enhance neutrophils adhesion to alveolar epithelial cells (A549), presumably in part by stimulating Rho/Rho-associated kinase (ROCK) signal-mediated actin cytoskeleton reorganization associated with thyrosine phosphorylation of focal adhesion kinase (FAK) [190].

Termination of the signal

Proteases activate PARs by an irreversible mechanism: cleavage exposes a tethered ligand domain that would be constantly available to interact with the receptor. Activation would then result in prolonged signalling unless there were efficient mechanisms to attenuate the response. Although the molecular mechanisms and pathways of agonist-induced trafficking of PARs can vary from receptor to receptor and in different cells [73], some common factors seem to apply.
The intracellular signal following PAR activation can be terminated by **receptor desensitization**, which takes place by uncoupling of the G proteins from the intracellular domain of the cleaved and activated receptor. In addition to the desensitization process that shuts off an already activated signalling pathway, the cells can also determine their responsiveness to agonists by regulating the levels of receptors that are expressed at the plasma membrane and which are thus accessible to agonists in the extra cellular fluid. Accordingly, **down regulation of receptor expression** is a second way of terminating a signal by rendering the cells non-responsive to the agonist.

The desensitization mechanism that terminates signalling by PARs is broadly similar to the classical pathway of desensitization that has been described in detail for many other GPCRs (particularly rhodopsin and the β_2-adrenergic receptor) [191]; ligand occupation of the GPCR induces the translocation of members of the family of G protein receptor kinases (GRKs) from the cytosol to the activated receptor at the cell surface. GRKs are serine-threonine kinases that phosphorylate activated GPCRs, usually within the carboxy terminus or third intracellular loop. This phosphorylation triggers

the membrane translocation of arrestins which interact with the phosphorylated GPCR, disrupt association with heterotrimeric G proteins, and thereby terminate signal transduction. Besides GRKs, this desensitizing C-tail phosphorylation can be mediated by other protein kinases like protein kinase A (PKA) and protein kinase C (PKC), although this process seems not to involve β-arrestin recruitment to the receptor [192, 193]. However, there remain many critical aspects of desensitization of GPCRs, including PARs, which are unexplored. In many cases, the GRK and arrestin isoforms that are co-expressed with receptors in question and which mediate desensitization to physiological stimuli are largely unknown. Mechanisms of desensitization also vary between different PARs, probably due to structural differences, especially in the intracellular loop 2 and carboxy terminus.
With regards to PAR_2, phosphorylation at serine and threonine residues in the C-tail domain is shown to distinctly regulate and to be critical for receptor desensitization, β-arrestin binding, and endocytic trafficking [170, 194]. The specific kinases responsible for C-tail phosphorylation of activated PAR_2 is not identified, but PKC appears to play a role in regulating the PAR_2 signalling [74, 170]. Activation of PKC abolishes PAR_2-induced Ca^{2+} signalling in transfected cell lines and enterocytes that naturally express this receptor, whereas PKC inhibitors magnify responses to PAR_2 activators. Mutation of a putative PKC site in the carboxy tail of the receptor renders it unresponsive to inhibition by active PKC [170].

The level of expression of receptor at the cell surface is a balance between removal by endocytosis and replenishment by recycling or mobilization of intracellular pools. PARs, which once cleaved, cannot be reused by the cell and are destined for intracellular degradation once internalized by endocytosis. This process will irrevocably terminate the signal and recovery requires synthesis or mobilization of new receptors.
PAR_2 activation is shown to induce translocation of β-arrestins to the plasma membrane where they interact with PAR_2 to mediate both desensitization and endocytosis [170, 195]. Endocytosis of PAR_2 proceeds by a clathrin-mediated mechanism and the activated PAR_2 is trafficked to lysosomes for degradation [66, 74, 195]. The importance of β-arrestins in endocytosis of PAR_2 is investigated by the use of mutated β-arrestin (mutation comprises the clathrin binding domain) that prevents endocytosis, and a mutated PAR_2 that fails to internalize due to its inability to interact with β-arrestins in transfected cells [89, 194]. In mouse embryonic fibroblasts deficient in β-arrestin 1 and 2 expression, PAR_2 desensitization was significantly impaired and internalization of PAR_2 virtually abolished compared to wild-type β-arrestin expressing cells [180]. Thus, PAR_2 appear to belong to the class B GPCRs that form high-affinity interactions with arrestins although phosphorylation-deficient PAR_2 mutants has been shown to be capable of constitutive internalization independent of β-arrestins [194]. The adaptor protein that mediates this constitutive β-arrestin-independent internalization is not known. Furthermore, Ramachandran and co-workers (2009) have shown that PAR_2 mutated at the two first amino acids in the tethered ligand sequence ($rPAR_2$-Leu^{37}-Ser^{38} and $rPAR_2$-Ala^{37-38}) fails to interact with both β-arrestin-1 and -2 when activated by trypsin, resulting in diminished internalization. On the contrary, when activated by the PAR_2 agonist peptide SLIGRL-NH_2, both receptor mutants interacted with both β-arrestins and displayed normal internalization compared to wild type PAR_2. This shows that the ability to interact with β-arrestins also shows some functional selectivity depending on the agonist that activates the

receptor, as previously described for intracellular Ca^{2+} signalling and MAP kinase activation (trypsin activates MAP kinase in both mutants, but not intracellular Ca^{2+} signalling).
Although the molecular mechanisms that target PAR_2 for degradation are largely unknown, ubiquitination of some other GPCRs is a prerequisite for degradation [196]. PAR_2 is shown to be extensively monoubiquitinated at multiple sites by the E3 ligase c-Cbl and targeted to lysosomes by an Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate)-dependent pathway after activation [197, 198]. Additionally, it is recently shown that endosomal deubiquitinating proteases (DUBs) are key regulators of PAR_2 down-regulation [199]. For a detailed review of the endocytic trafficking and degradation of PARs the readers are referred to Soh *et al* 2010 [66].

Tissue expression of PAR_2

A myriad of physiological and pathophysiological events have been attributed to PAR-mediated signalling within the last 16 years since their identification. PARs are widely expressed in tissues from numerous organ systems and seems to comprise important physiological and pathophysiological roles in the cardiovascular and circulatory-, immune- , nervous- , gastrointestinal- , and renal system in addition to the respiratory system. Their specific involvement in various processes remain however unknown and there are several obstacles to understanding the complete role of PARs [47, 73]. First; there are a complex interplay within PARs, the multiple PAR activating proteases and disabling proteases, protease inhibitors, co-factors or anchoring proteins, and other molecules that may be present in different organ systems, and under different pathological conditions. This makes the understanding of PAR-mediated events a complex exercise. Secondly; most studies rely on administration of activators (proteases or agonist peptides). Although such studies provide information about the potential role of PARs, they do not provide direct information about their function in physiology or disease states. Proteases are not selective activators of PARs, and agonist peptides are agonists at only very high concentrations. Peptides at high concentrations can have biological effects that are unrelated to PAR activation. Finally; selective antagonists of PARs are not widely available. In the absence of selective activators and antagonists, the best current approach to specifically determine the role of PARs *in vivo* is to study genetically modified animals. Mice have been developed that lack all known PARs [200-205], but the use of genetically modified mice is also fraught with problems. Effects of deletion on embryonic survival and fertility may impede the breeding of animals for experimentation. These facts have to be kept in mind when current knowledge regarding PARs is assessed.
PAR_2 is expressed ubiquitously by barrier cells of a variety of organs (i.e. epithelia and endothelia). The epithelial expression of PAR_2 is particularly striking and has led authors to suggest that PAR_2 may be involved in defensive reactions at these barriers [108, 109, 206, 207]. To highlight the prevalence and multifaceted functions of PAR_2 a short summary of the expression and function of this receptor in different organ system is given below before a more detailed resumé of the existing information regarding PAR_2 in the airways is provided.

PAR_2 in the cardiovascular and circulatory system

PARs are expressed by multiple cells in the cardiovascular and circulatory system, and PAR_2 expression is detected both on endothelial cells [208, 209] and vascular smooth muscle cells [210, 211]. Activation of endothelial PAR_2 (rat/porcine/human) causes vascular relaxation [76, 212, 213]. PAR_2 activation can also cause proliferation of cultured vascular smooth muscle [214, 215] and endothelial cells [209], and an *in vivo* murine model shows stimulation of angiogenesis after PAR_2 activation [216]. Additionally, inflammatory agents (TNFα, IL-1, LPS) and tissue damage is found to up-regulate PAR_2 in the vasculature [217-220].

PAR_2 in the immune system

PAR expression is revealed on many immune cells and the primary response mediated by PARs in the immune system appears to be related to chemotaxis and cytokine release from inflammatory cells. PAR_2 is detected on mast cells [221], eosinophils [222], monocytes [223], macrophages [223, 224], and neutrophils [225-227].

The function of PAR_2 activation in these cells is not always clear, but eosinophil PAR_2 activation results in degranulation and superoxide production [222, 228]. Exposure to mast cell tryptase induces eosinophil IL-8 release [229], while stimulation with trypsin and PAR_2 agonist peptide triggered shape change and release of cysteinyl leukotrienes [228]. Neutrophil PAR_2 activation causes shape changes and enhanced CD11b/CD18 expression [225], and PAR_2 agonists is also shown to cause enhanced neutrophil motility in 3D collagen gel lattices through up-regulation of cell surface integrins [227].

PAR_2 in the nervous system

The expression of all four PARs has been detected in CNS of rodents [230]. PAR_2 expression has been found on normal human astrocytes and neurons [210], rat spinal cord and isolated dorsal root ganglia [231], and guinea pig myenteric [120] and submucosal neurons [232].

A majority of the research involving PARs in the nervous system deals with PAR_1 and PAR_2 is to a lesser extent investigated. PARs in the central nervous system are shown to influence upon the astrocyte morphology and proliferation, and morphology and outgrowth of neurites in nerve cells [47]. Studies on PAR_2 function in the peripheral nervous system have pointed to a role for these receptors in neurogenic inflammation [125], hyperalgesia, analgesia [126] and itching [233], as well as playing roles in nerve regeneration [234], gastric epithelial ion secretion [235] and mucous secretion function [236, 237].

PAR_2 in the gastrointestinal system

Of all the body systems, the gastrointestinal tract is most exposed to proteases, both from physiologically relevant processes such as digestion and from exposure to the bacterial flora of the gut. All of the PARs have been found to be expressed in the gastrointestinal tract and PAR activation is involved in ion transport, permeability, motility and inflammation [47]. PAR_2 expression is detected on enterocytes [127], epithelial cells [97], smooth muscle cells [76], endothelial cells [238], colonic myocytes [127], myofibroblasts [239], and neurons [232].

PAR_2 in the renal system

Initial PAR studies showed abundant expression of PAR_2 mRNA in the kidney [71] and further work has documented the presence of PARs widely distributed throughout the kidney [202, 240]. PAR_2 activation has been found to cause vasodilatation in a perfused rat kidney model [240, 241] and mediation of mesangial cell proliferation (by factor Xa activation) [242].

PAR_2 in the airways

Distribution of PAR_2

PAR expression is detectable in a wide range of airway cells, and evidence so far suggests that PARs play critical roles in pulmonary physiology and pathology. Although human PAR_2 mRNA was found to be highly expressed in cultures of A549 cells (a human lung epithelial cell line) [97], the first evidence that PAR_2 protein was expressed in the intact lung was provided by D'Andrea and co-workers (1998) a few years later [210]. PAR_2 expression is detected on ciliated and non-ciliated epithelial cells, especially at the apical membrane [42, 44, 108, 150, 210, 243], glands [244], smooth muscle cells [108, 210, 245, 246], endothelial cells [210, 246], fibroblasts [57, 113, 247], and the mesothelial cells of the pleura [248]. In human foetal lung tissue, marked PAR_2 immunoreactivity is also observed in cuboidal type II-like epithelial cells [110].

Function of PAR_2

As in most other tissues, the physiological agonists of PAR_2 in the airways remain to be determined. All proteases that are experimentally shown to activate PAR_2 are listed in the previous chapter, but a vast number of these are exogene proteases and yet others are proteases not encountered in the airway tissue. Potential endogen airway PAR_2 agonists are mast cell tryptase [122] and human airway trypsin [113]. Neutrophil proteases (proteinase 3, cathepsin G, neutrophil elastase) are in numerous sources indicated as possible PAR activators in addition to their confirmed role as PAR_2 inactivators [151, 152], but so far PAR_2 activation has not been confirmed with certainty.

An important function of PARs in the respiratory system involves the regulation of **airway tone** by causing either a contraction or relaxation of smooth muscle cells [249]. Their involvement has also been demonstrated in **remodelling of the lung**, through promoting secretion of pro-inflammatory and pro-fibrotic mediator release [250], the production of extra-cellular matrix components and through stimulating cell mitogenesis [251]. PARs may also regulate the **inflammatory response** in the airways through recruitment of inflammatory cells [109, 249]. The effects attributable to PAR_2 in these conditions are summarized below.

Airway tone

PAR_2 plays an important role in regulating airway tone with studies showing both protective bonchodilatation as well as detrimental bronchoconstriction. Epithelial PAR_2 activation in isolated bronchi results in **relaxation** in mice [108], rats [108, 252], guinea pigs [108], and humans [108]. Relaxation also occurs in isolated mouse and guinea pig trachea and was found to be mediated by prostaniods in mice, particularly PGE_2 [253-255]. As opposed to these reports, activation of PAR_2 in isolated human bronchi and second order intrapulmonary bronchi from guinea pig leads to **constriction** following administration of PAR_2-AP [246, 255].

In addition to these diverging results in airway tone collected from tissue preparations, the results from *in vivo* studies in different animal species are also contradictory. *In vivo* studies in rats show that aerosol administration of PAR_2-AP **inhibits** serotonin-induced bronchoconstriction [108], while *in vivo* studies conducted in mice reports that intranasal administration of PAR_2-APs **causes** bronchoconstriction [204]. These observations could be due to inter-species differences; however, *in vivo* results originating from the same species (guinea pig) are also inconsistent. Intravenous administration of PAR_2-AP is shown to inhibit histamine-induced increase in airway resistance by a mechanism independent of the release of prostaglandins, nitric oxide or the effect of circulating adrenaline [256]. In contrast, intravenous and intra-tracheal administration of PAR_2-AP causes bronchoconstriction, an effect mediated in part by prostanoids that involves the release of tachykinins from sensory nerve endings [255].

The conflicting observations concerning airway tone could be due to a dose-repose relationship yielding dissimilar responses according to the administered doses; however, the concentrations of peptide agonist used do not diverge much. A possible explanation for this discrepancy is that PAR_2 activation results in relaxation in the main bronchi and the trachea, but causes a contractile response in tissues isolated from smaller intrapulmonary bronchi [255]. Thus, PAR_2 may be protective in the larger airways, but activation in the smaller bronchioles may increase airway resistance, an effect that might be detrimental in the intact animal as the smaller bronchioles are the principal site of airway resistance [47, 70]. Finally, although the use of agonist peptides in PAR-related research usually yields receptor specific results, some of the observed effects of proteases on airway resistance could be independent of activation of PAR_2. For example, trypsin can evoke contraction of isolated guinea pig bronchus, whereas PAR_2-AP and tryptase do not evoke contraction [257].

Cellular effects

In addition to the regulation of airway tone, PAR_2 is shown to be involved in a variety of cellular effects like growth and proliferation, migration, secretion of inflammatory mediators, and disruption of cellular adhesions; all factors that may contribute to the initiation and progression of -, as well as accompany inflammation.

Activation of PAR_2 is shown to induce proliferation of smooth muscle cells [118, 245, 258], epithelial cells [259], and fibroblasts [57, 113]. Cellular growth and proliferation may lead to airway remodelling and fibrosis in addition to regeneration and reparation of damaged tissues.

Activated PAR_2 also leads to migration of inflammatory cells into the airway tissue. Berger and co-workers (1999) have demonstrated increased mast cell infiltration into subepithelial tissue in isolated human bronchi following mast cell tryptase stimulation, an effect thought to be acting via PAR_2 [260]. Macrophage and eosinophil infiltration into the airway is also shown to occur following PAR_2 activation [204]. Infiltration of inflammatory cells will increase the affected tissues responsiveness to activating mediators. *In vivo* studies in mice show increased airway hyperresponsiveness and inflammatory cell infiltration in response to PAR_2 agonist administration. Additionally, these effects are shown to be lowered if the receptor is lacking as there are observed reduced eosinophil infiltration, reduced airway hyperreactivity and reduced IgE levels to ovalbumin in PAR_2-deficient mice [204, 261]. On the contrary, both Moffatt and co-workers (2002) and De Campo and Henry (2005) found that intranasal delivery of PAR_2-AP in mice did not cause airway inflammation, but instead inhibited the marked immune cell inflammatory response (infiltration of polymorphonuclear leukocytes) triggered by LPS administration [262] and inhibited the development of eosinophilia and hyperresposiveness following ovalbumin challenge [263].

These diverging results presenting both pro- and anti-inflammatory effects reflect what is found in the studies regarding airway tone, where PAR_2 in many instances displays a negative contractile effect, but in other studies seems to have a protective relaxant effect. However, it is to be noted that these conflicting reports used different concentrations of the PAR_2-AP for stimulation; the researches that report pro-inflammatory effects had stimulations in the µg-scale (160 µg/kg for Schmidlin *et al* 2002 [204] and around 80 µg/kg for Ebeling *et al* 2005 [261]), while the anti-inflammatory effects were observed using stimulations in the mg-scale (25 mg/kg for Moffatt *et al* 2002 [262] and De Campo and Henry 2005 [263]). The anti-inflammatory effects following PAR_2 activation is linked to the secretion of PGE_2. As concluded by Chignard and Pidard (2006), these diverging results might perhaps be due to differential production of PGE_2 when PAR_2 is activated by high or low peptide concentrations [264].

Furthermore, PAR_2 is shown to be involved in the release of different mediators from cultured airway cells. Activation of PAR_2 is shown to mediated release of MMP-9 [182], IL-6, IL-8 and PGE_2 [42] from human airway epithelial cells (A549, SAEC (small airway epithelial cells)), and macrophage inflammatory protein (MIP)-2 from murine mesothelial cells [248]. Allergen stimulated airway epithelial cells (A549) is shown to release GM-CSF and eotaxin, an effect possibly mediated by PAR_2 [149, 243], and recent reports have also suggested a role for PAR_2 in stimulating cytokine production in cultured airway fibroblasts [247].

Additionally, activation of PAR_2 is shown to influence upon the contact that exist between epithelial cells, a function that is essential in maintaining the integrity and homeostasis of intact, healthy tissue. A report by Winter and co-workers (2006) describes that PAR_2 activation interrupts E-cadherin adhesion and compromises the airway epithelial barrier that divides the epithelium into functionally restricted apical and basolateral domains [265]. Furthermore, PAR_2 is also suggested to have a role in stimulating adhesion molecule expression in cultured airway fibroblasts [247], an effect that might influence upon infiltration of neutrophils during an inflammatory response.

Contribution to physiological and pathophysiological mechanisms in the inflammatory response

The specific physiological functions of PAR_2 in the airways and other organ systems are currently a topic under investigation. Existing information points to a dual role with both pro- and anti-inflammatory effects of receptor activation and the role of PAR_2 in pulmonary biology seem to be particularly intriguing. The pro- and anti-inflammatory effects caused by receptor activation do not have to be mutually exclusive. Inflammation is essentially a protective response intended to eliminate both the initial cause of cell injury and the necrotic cells and tissues arising as a consequence of such injury. As such, inflammation is also intimately interwoven with protective mechanisms and repair processes. For the inflammation to be beneficial it has to be confined to the damaged area and self-limiting. In order to accomplish this, protective mechanisms as well as tissue repair processes must be activated and the inflammatory response terminated when the injurious stimulus is removed. The possibly dual role observed for PAR_2 may reflect this complex relationship. Activation of PAR_2 by endogenous agonists may be a part of the physiological regulation of both pro- and anti-inflammatory effects, while in situations where PAR_2 is activated by excessive amounts of exogenous proteases this equilibrium may be interrupted and the pro-inflammatory qualities becomes more prominent. In addition to the frequently discussed effects on inflammation, PAR_2 is found to be involved in electrolyte transport across airway epithelial cell membranes. The composition of electrolytes may also have impact on airway inflammatory processes as imbalances in the secretion/absorption of ions can influence the airway surface liquid volume, an important component of the mucociliary clearance machinery.

Inflammation

Infection or injury to the body results in inflammation. During inflammation and trauma, proteases from the coagulation cascade and from inflammatory cells could regulate pulmonary cells by activating PARs. There are elevated levels of coagulation proteases [266] and tryptase [267] in bronchoalveolar lavage from patients with chronic inflammatory diseases. PAR_2 (in addition to PAR_1 og PAR_4) have been implicated in mediating the vascular responses in inflammation like increased leukocyte endothelial adherence and rolling, as well as migration of leukocytes from vasculature into inflamed tissue. The increased expression of proteinases from inflammatory cells and from activation of the coagulation cascade very likely serves as triggers for the PARs in this type of setting [47].

In vivo studies conducted in rat shows that intraplantar administration of PAR_2-AP causes oedema and granulocyte infiltration from the vasculature [268]. The oedema is found to be dependent on the release of neuropeptides from sensory nerves, whereas the extravasation of granulocytes is believed to be due to a direct effect of activating PAR_2 on endothelial cells. Activation of PAR_2 on pleural mesothelial cells in mice results in chemokine release and neutrophil recruitment into the pleural cavity, indicating that PAR_2 plays a role in promoting pleural inflammatory responses [248]. Inflammation is usually accompanied by hyperemia. PAR_2 agonists are shown to dilate blood vessels in the human arm by mechanisms that include nitric oxide and prostaglandins, leading to hyperemia [269].

Although PAR_2 is found to be expressed on a majority of immune cell types, the function in these cells is not always clear. The primary response mediated by PARs in the immune system appears to be related to cytokine release from inflammatory cells and chemotaxis. PAR_2 activation results in IL-6, IL-8, and cysteinyl leukotriene release from-, shape changes in- and degranulation of eosinophils [222, 228, 229]. Shape changes and enhanced motility are also observed in neutrophils [225, 227]. Furthermore, PAR_2 is shown to be involved in leukocyte chemotactic migration by directing localized actin filament severing and membrane protrusions [173].

Chemokines released from airway cells induces inflammatory cells to leave the bloodstream and enter into the surrounding lung tissue. Cultured human airway epithelial cells are shown to secrete IL-8 following PAR_2 activation [42] and in cultured murine mesothelial cells PAR_2 agonist peptide stimulate release of macrophage inflammatory protein (MIP)-2 [248]. Yagi and co-workers (2006) report that epithelial PAR_2 stimulation enhance neutrophil adhesion to alveolar type II epithelial cells (A549), an effect that seems to be mediated by reorganization of the actin cytoskeleton in the epithelial cells [190]. Recently it has been reported that PAR_2 cooperates with toll-like receptors (TLRs). This implies a previously under-appreciated role of PARs as an additional level of the innate immune defence, apart from classical pattern-recognition receptors [159]. Additionally, proteases are shown to induce production of thymic stromal lymphopoietin (TSLP) in a human airway epithelial cell line (BEAS-2B) through PAR_2. By promoting TSLP production in the airways, proteases may facilitate the development and/or exacerbation of Th2-type airway inflammation, a process that seems to involve PAR_2 [270].

Chronic inflammation of mucosa is associated with an increased cancer risk and tumorigenesis in mucosal tissues is associated with the activity of some proteases, COX-2, and β-catenin [185]. Serine proteases may participate in inflammation and tumorigenesis through the activation of PAR_2. In human lung epithelial cells (A549), PAR_2 activation is shown to increase COX-2 expression via an ERK 1/2-mediated activation of the β-catenin/T cell factor-4 and CREB pathways [185]. These findings by Wang and co-workers (2008) [185] reveal new cellular mechanisms by which serine proteases may participate in tumor development and confirm the importance of PAR_2 in conditions of chronic inflammation and tumorigenesis.

Cytoprotection

The amount of studies indicating that PAR_2 is involved in a vast number of pathological conditions is increasing. Although our attention is focused on the pro-inflammatory qualities of PAR_2, there is also existing information that links the receptor to protective processes. The best evidence for PAR_2 being involved in normal physiological processes is cytoprotection in the pancreas and upper small intestine since these organs are episodically exposed to high concentrations of pancreatic trypsin [206]. PAR_2 is abundantly expressed on epithelia in the pancreatic duct [106] and intestine [104] and its activation leads to a range of cytoprotective effects driven largely by release from the epithelia of the secondary mediator prostaglandin E_2 (PGE_2) [206]. A similar PAR_2 and prostanoid-dependent cytoprotective system, as seen in the gut, might operate in the bronchial epithelia of the airways [108, 206]. A number of the effects produced by PAR_2 activators within the airways may be mediated by PGE_2 [271]. Although the potential involvement of PGE_2 in these effects has not fully been determined, PAR_2-mediated generation of

PGE_2 appear to have functional consequences in relation to the production of bronchoprotective effects like bronchodilation, inhibition of recruitment and activation of mast cells and eosinophils, as well as inhibitory effects on fibroblasts, possibly reducing airway remodelling [271, 272]. Airway epithelium is shown to release PGE_2 following PAR_2 activation [42], but the net effect of this secretion is not determined as PGE_2 also exhibits pro-inflammatory effects. Depending on the context, PGE_2 may induce fever, increase vascular permeability and vasodilation, and enhance pain and oedema caused by other agents [273] as well as mediating the anti-inflammatory effects previously described.

However, pro- or anti-inflammatory properties, the source of the naturally-occurring enzyme activator of airway PAR_2 under these possible cytoprotective events need careful consideration. Mast cell tryptase (MCT) is known to activate PAR_2 [98, 122], although less effectively than trypsin. MCT has been reported to be a mitogen for a variety of cells involved in airway wall remodelling [206] and can cause airway hyper-responsiveness [55, 260] in isolated bronchi (guinea pig, human). PAR_2 is by many thought to be the molecular target central to these effects giving it a pro-inflammatory role. It is likely that MCT is a genuine PAR_2 activator on many airway cells, but some space-distance limitations have been commented. Because airway epithelia mostly express PAR_2 on their apical surfaces [108] and the population of intraepithelial mast cells is confined to the most basal regions of the airway epithelium [274], it is difficult to envisage MCT being able to transverse the molecular sieve of tight junctions between epithelial cells that separate the airway lumen from the submucosa to activate apically restricted PAR_2 under normal conditions. In asthmatic patients, on the other hand, the epithelial disruption is widespread [275] and MCT can be detected in bronchoalveolar lavage (BAL) fluid [267]. Though, it might be a possibility that PAR_2 is positioned apically in order to detect MCT during perturbations of the epithelial barrier and such being a part of the airway cytoprotective system. On the other hand, the up-regulation of PAR_2 expression detected in asthmatic patients [44] may also contribute to pro-inflammatory events. Until we have knowledge about all physiological activators and target signalling pathways of PAR_2 it seems to be a difficult exercise to determine the exact effects of receptor activation in vivo.

An alternative to the hypothesis that MCT is the primary PAR_2 activator in the airways is tryptic enzymes secreted at the apical pole of epithelial cells under “normal” circumstances, like human airway trypsin [109, 113]. The demarcation between the luminal and subepithelial compartments by tight junctions, allows trypsin and MCT to have access to different molecular targets. The widespread distribution of PAR_2 might signify that this receptor acts as a sensor for a variety of enzymes with different idiosyncrasies and distinct extracellular environments, playing different roles in homeostasis (pro-inflammatory vs. anti-inflammatory) in each case [109].

Electrolyte transport

PAR_2 is also found to regulate ion transport in mouse and human airway epithelial cell cultures [121, 276, 277], a mechanism that may control fluid volume and composition at the airway surface through regulation of basal Na^+ reabsorption and both basal and stimulated anion secretion [70]. PAR_2 appear to be expressed on both the apical and basolateral sides of the epithelium, however only basolateral

stimulation resulted in inhibition of Na^+ conductance and stimulation of both luminal Cl^- channels and basolateral K^+ channels [121, 276]. A delicate balance between sodium (Na^+) absorption and chloride (Cl^-) secretion is necessary to optimize the volume of airway surface liquid, this in order to promote ciliary activity and mucociliary clearance, effectively removing inhaled particles. Stimulation of PAR_2 may cause accumulation of airway surface liquid, a process that may help to flush noxious stimuli away from the affected airways.

PAR_2 in mechanisms of airway disease

Proteases and PARs make important contributions to a variety of diseases. First; PARs regulate many biological processes that are critical in disease, including coagulation, proliferation and survival, inflammation, neurotransmission, and pain. Second; proteases that activate PARs are generated during diseases, for example, in trauma, hemostasis, inflammation, and tumor formation. Finally; the expression and function of PARs are altered in disease [73].

Many cell types known to play important roles in both the healthy and diseased airways express functional PAR_2. Studies in animal models have shown that altering the level of PAR_2 expression or administering exogenous PAR_2 activators can significantly change the course of several airway diseases. Furthermore, some respiratory tract diseases have been associated with altered levels of PAR_2 expression and function [271]. PAR_2 is generally found to be up regulated during inflammation [220, 278], and lung inflammation induced by lipopolysaccharide [279] or influenza A virus [280] is also associated with increased expression of PAR_2. Levels of immunoreactive PAR_2 staining in the epithelium of bronchial biopsy specimens obtained from asthmatic subjects were significantly higher than non-asthmatic subjects [44]. PAR_2 is also found to be highly expressed in airway epithelium of preterm infants who died of prolonged respiratory distress syndrome or of bronchopulmonary dysplasia. The level of PAR_2 expression tended to increase with progression of respiratory distress syndrome toward bronchopulmonary dysplasia [110].

Not all pulmonary insults are associated with increased PAR_2 expression. Smoking does not appear to affect levels of PAR_2 as expression was found to be similar in the central airways of smokers and non-smokers (bronchial smooth muscle, epithelium, glands, vascular endothelium, vascular smooth muscle, alveolar macrophages) [224, 244]. However, when smokers were divided according to the presence of symptoms of chronic bronchitis or chronic airflow limitation (COPD), PAR_2 expression was increased in vascular smooth muscle of smoking patients with bronchitis compared to smoking patients with COPD [244].

Neutrophil derived proteases such as elastase and cathepsin G are capable of disarming PAR_2 most likely by proteolysis of the extracellular domain downstream from the trypsin cleavage/activation site [151]. Elastase (an elastolytic metalloprotease) from *Pseudomoas aeruginosa*, a major pathogen in cystic fibrosis, is also shown to disable PAR_2 in respiratory epithelial cells. This might alter the host's innate defence mechanisms and respiratory functions, thus contributing to the disease pathology in cystic fibrosis [153]. Using a murine model of acute *P. aeruginosa* pneumonia, Moraes and co-workers

(2008) have examined differences in the pulmonary inflammatory response between wild-type and PAR_2 (-/-) mice. Compared with wild-type mice, PAR_2(-/-) mice displayed more severe lung inflammation and injury in response to *P. aeruginosa* infection as indicated by higher bronchoalveolar lavage (BAL) fluid neutrophil numbers, protein concentration, and TNFα levels. They conclude that genetic deletion of PAR_2 is associated with decreased clearance of *P. aeruginosa*, and the data suggest that a deficiency in IFNγ production and impaired bacterial phagocytosis are two potential mechanisms responsible for this defect [281].

There has been considerable interest in the role of tryptase in airway inflammation and asthma. Hyperplasia of smooth muscle contributes to remodelling of the airways that accompanies asthma, and tryptase induces proliferation of airway smooth muscle cells, fibroblasts, and epithelial cells [259, 282, 283]. Tryptase also induces hyperresponsiveness in human isolated bronchi [260]. Similar effects on cellular proliferation and airway hyperresponsiveness is produced by PAR_2 agonist peptides [57, 245]. A key step in activation of human airway smooth muscle cells is an increase in free intercellular Ca^{2+} concentration. Tryptase induces an increase in intracellular Ca^{2+} that is significantly inhibited by the use of siRNA against PAR_2 [117], indicating that airway hyperresponsiveness in fact is mediated by tryptase activation of PAR_2. Animal studies have shown that selective inhibition of tryptase and serine protease inhibition blocks airway inflammation in mouse asthma models, reducing total cells, eosinophils and lymphocytes in BAL fluid, airway tissue eosinophilia, goblet cell hyperplasia, mucus secretion, and peribroncheal edema in addition to an inhibition of IL-4, IL-13, and eotaxin release in BAL fluid [284, 285]. These findings suggest that increased serine protease activity in the airways is involved in the development of allergic eosinophilic inflammation and epithelial remodelling in bronchial asthma, effects possibly mediated by PAR_2 activation. Furthermore, ovalbumin-induced infiltration of eosinophils and increased eotaxin content is shown to be abrogated in BAL fluid from PAR_2 (-/-) mice, indicating that activation of PAR_2 might be essential in the production of eotaxin and consequential allergic inflammation in the airways [286].

An imbalance between protease and antiprotease activity in the lung is proposed as the major mechanism resulting in emphysema [287]. Neutrophil elastase is more than a matrix-degrading enzyme, and is considered to have potential multiple roles in the pathogenesis of both emphysema and lung fibrosis [288]. α_1-antitrypsin is the natural inhibitor of neutrophil elastase and low levels of α_1-antitrypsin is associated with COPD [289], asthma [290], and bronchiectasis [291]. Although it is not established that PAR_2 is involved in the pathological mechanisms caused by neutrophil elastase, it is a serine protease with the potential of being a PAR_2 activator in addition to the demonstrated capacity to inactivate PAR_2 [151]. A deficiency in the natural protease inhibitor would influence on the balance between protease and antiprotease resulting in a cellular environment displaying increased protease activity possibly leading to PAR_2 activation.

Therapeutic implications

The observation that inflammation is diminished in PAR-deficient animals suggests that PAR antagonists and protease inhibitors may be useful anti-inflammatory agents [73]. Developing PAR_2 antagonists have presented a great challenge with very limited success so far. Largely because of (1) the difficulty in synthesizing serine protease inhibitors that are enzyme selective and (2) the ability of several serine proteases to activate PARs $_2$ and $_4$, the receptor-activating proteases themselves do not appear to be attractive therapeutic targets for blocking the activation of these two PARs [47].

The most promising results so far is with compounds that targets the receptor; **PAR_2 antagonists**. Some success has been achieved with antagonists based on modified PAR_2 tethered ligand peptides (FSLLRY-NH_2, LSIGRL-NH_2), but the potency are too low to be considered for general systemic use [292]. These peptides are able to inhibit trypsin activation of PAR_2 through possible interactions with the tethered ligand docking site, but are of limited use to block activation of PAR_2 by PAR agonist peptides. The peptide, LIGK-NH_2 is found to block both trypsin and PAR_2 agonist peptide activation of PAR_2 and a peptidomimetic based on this peptide sequence, ENMD-1068 (N1-3-methylbutyryl-N4-6-amino-hexanoyl-piperazine), has proved of use in studies done *in vivo*, despite a very low potency [293]. Although ENMD-1068 can serve as a "lead" compound, the synthesis of high potency orally available PAR_2 antagonists is a goal yet to be reached.

PAR_2-targeted antibodies (polyclonal B5, monoclonal SAM11) and PAR_2-targeted siRNAs may prove of therapeutic value, but the methods is not sufficiently developed to be considered for therapeutic use as of yet [293].

Although the PAR-activating proteases are challenging therapeutic targets, protease inhibition by **protease inhibitors** in a restricted environment may have value. The serine protease-dependent, PAR_2 mediated inflammation caused by *Citrobacter rodentium* in a murine model of colitis can be attenuated by the oral administration of the soya trypsin inhibitor [294]. Additionally, the systemic administration of protease inhibitors in a murine model of joint inflammation is able to diminish joint swelling significantly [293].

The fact that different proteases can activate the same PAR to elicit distinct signalling responses suggests that different drugs could be developed to target PAR signalling selectively. In order to design drugs to meet these expectations we must achieve more knowledge about the signalling and cell type-specific responses elicited by different proteases, as well as receptor interactions and functional selectivity.

Selected signalling molecules

Cytokines

A hallmark of inflammation is the recruitment of neutrophils from the blood to the injured tissue, a process directed by chemotactic cytokines called chemokines. About 40 human chemokines are known today with IL-8 as the founding member of this superfamily [295, 296]. Effects from IL-8 secretion involve three main changes in neutrophils; (1) shape changes and directional migration, (2) exocytosis of storage proteins, and (3) the respiratory burst [295]. One of the most remarkable properties of IL-8 is the variation of its expression level. Normally, IL-8 protein is barely secreted from non-induced cells, but its production is rapidly induced by a wide range of stimuli encompassing proinflammatory cytokines like tumor necrosis factor (TNF) or IL-1 [297, 298], bacterial [299, 300] or viral products [301, 302] and cellular stress [303-306]. IL-8 can be synthesized by a variety of cell types including human pulmonary epithelial cells (A549) [186].

Mitogen activated protein kinases

Protein kinases participate in protein phosphorylation cycles where the kinase covalently links a phosphate group to the substrate generating a phosphorylated target protein [64]. The members of the mitogen activated protein (MAP) kinase family are protein serine/threonine kinases controlling key cellular functions including gene transcription, protein synthesis, proliferation, differentiation, migration, embryogenesis, and apoptosis. These kinases are shown to participate in a diversity of physiological and pathological conditions like embryonic development, metabolism, learning, innate and acquired immunity, chronic inflammation, heart disease, diabetes mellitus, and cancer [307, 308].

Four MAP kinase cascades have so far been defined in mammals; the extracellular signal-regulated kinase (ERK 1/2), the p38 kinases, the c-Jun N-terminal kinases (JNK 1/2/3), and the ERK5/BMK (BIG MAPK) [309, 310]. These different cascades respond to distinct extracellular stimuli. Activation of two of these cascades following trypsin exposure is explored in this work; 1) The extracellular signal-regulated kinase regulates differentiation, proliferation, and meiosis in response to mitogens like growth factors, cytokines, and phorbolesters. 2) The p38 kinases are involved in immunity and are activated by chemical and physical stresses like UV irradiation, bacterial lipopolysaccharides, ischemia, cytokines, osmotic shock, and heat shock. The two latter cascades (JNK 1/2/3 and ERK5/BMK) are not explored in the present study.

Transcription factors

NF-κB is a collective term referring to dimeric transcription factors that belong to the Rel family and are regulated via shuttling from the cytoplasm to the nucleus in response to cell stimulation [311]. Inactive NF-κB is present in the cytoplasm of all cells; only when it is activated and translocated to the nucleus is the activating sequence of events generated. Mammals express five Rel (NF-κB) proteins that belong to two classes. The first class includes RelA (p65), RelB, and c-Rel, proteins that are synthesized as mature products and do not require proteolytic processing. The members of the second group is synthesized as large precursors, p105 (NF-κB1) and p100 (NF-κB2), that require

proteolytic processing to produce the mature p50 and p52 NF-κB proteins. NF-κB dimers containing RelA or c-Rel are held in the cytoplasm through interaction with specific inhibitors, the IκBs. These inhibitory proteins undergo rapid ubiquitin-dependent degradation after exposure to a variety of agonists, which activate the IκB kinase (IKK) complex [312]. Activated NF-κB dimers then translocate to the nucleus and bind to their specific base pair site and regulate the expression of over 200 immune, growth, and inflammation genes including IL-8 gene expression [313]. This pathway of NF-κB activation is called the **classical (canonical) pathway**, see fig 3. The second major pathway for NF-κB activation; **the alternative pathway**, results in specific activation of p52/RelB heterodimers. The alternative pathway is not directly involved in innate immunity and inflammation, but is required for the generation of secondary lymphoid organs and for B-cell maturation and survival [314].

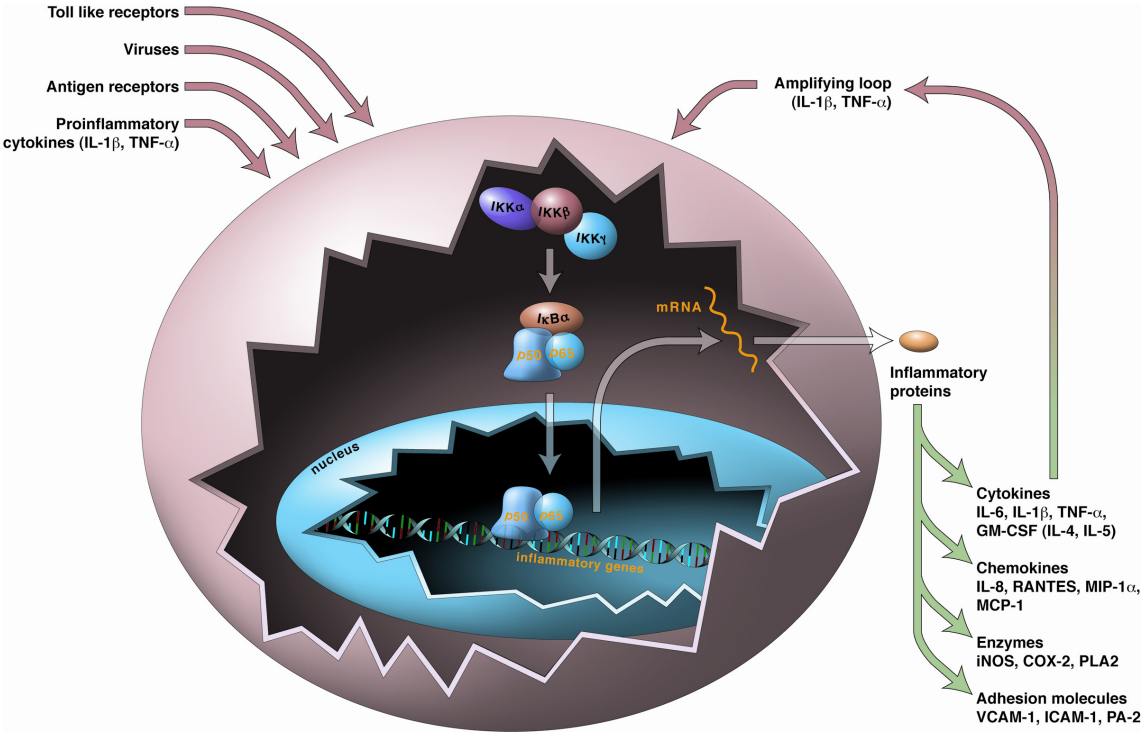

Figure 3

Classical NF-κB pathway

The classical NF-κB pathway is activated by a variety of inflammatory signals, resulting in coordinate expression of multiple inflammatory and innate immune genes. The proinflammatory cytokines IL-1β and TNF-α activate NF-κB, and their expression is induced in response to NF-κB activation, thus forming an amplifying feed forward loop. (COX-2) cyclooxygenase 2, (GM-CSF) granulocyte–macrophage-colony-stimulating factor, (ICAM-1) intercellular adhesion molecule 1, (IKK) IκB kinase, (IL-1β) interleukin-1β, (iNOS) inducible nitric oxide synthase, (MCP-1) monocyte chemotactic protein-1, (MIP-1α) macrophage inflammatory protein-1α, (PLA2) phospholipase 2, (TLRs) Toll-like receptors, (TNF-α) tumor necrosis factor α, (VCAM-1) vascular cell adhesion molecule-1.

Adapted from Bonizzi and Karin (2004), *Trends Immunol* 25: pp 280 – 288 [314]

Aims of the presented study

The aim of this study was to increase the understanding of serine proteases, PAR_2 and their respective significance in airway reactivity towards fish (salmon [*Salmo salar*], sardine [*Sardinops melanostictus*]) and crustaceans (king crab [*Paralithodes camtschaticus*]). We desired to achieve new knowledge about serine proteases originating from seafood and their ability to contribute to inflammatory reactions in human airways with emphasis on epithelial tissue. In order to illustrate our aim we set out to;

(1) Investigate whether trypsin from salmon can initiate inflammation in human airways by activating PAR_2 and find out which intracellular signalling systems are involved.

(2) Determine if trypsins from other fish species behave similar to salmon trypsin with respect to PAR_2 activation and secretion of inflammatory mediators from airway epithelial cells.

(3) Establish if king crab trypsin display similar capacity of activating PAR_2 as the fish trypsins, and if not, identify alterations in PAR_2 activating capacity or PAR_2 coupled intracellular signalling pathways that might reflect prospective differences.

Summary of papers

Paper I

Bang B, Larsen M, **Larsen AK**, Aasmoe L. (2009). Proteases from salmon stimulate IL-8 in airway epithelial cells. *J Toxicol Environ Health* A. 72 (9): pp 571 – 573

Bacterial derived endotoxin has often been regarded as the most important exposure agent linked to non-allergic airway inflammation in work environments with sources of biological exposures. However, there is now increasing awareness of the role of exogenous proteases and activation of PAR_2 in airway inflammation induced by biological agents. In this paper we have studied the ability of salmon tissue extracts from stomach, pyloric ceca, and skin mucus to stimulate IL-8 production in human airway epithelial cells. A549 cells were stimulated with various concentrations of fish tissue extracts for 6 hours before the amount of secreted IL-8 in the supernatant was determined. The role of serine protease enzymes and endotoxin in the tissue samples were investigated by the use of inhibitors. We show a concentration-dependent increase in IL-8 secretion after stimulation with the salmon tissue extracts, an effect most likely due to serine proteases in the tissue samples as the IL-8 stimulating effect was inhibited by serine protease inhibitors but not by endotoxin inhibitors. Tissue extract derived from the pyloric ceca was most potent in capacity of inducing an IL-8 response in the cell based assays and also displayed the highest trypsin-like activity determined by the hydrolyzation of DL-BAPNA. Regarding the high content of pancreatic enzymes in this specialized structure with trypsin being the most important, these findings were not unexpected. In conclusion, the data in this paper demonstrate that fish tissues contain inflammatory potential linked to serine protease activity.

Paper II

Larsen AK, Seternes OM, Larsen M, Aasmoe L, Bang B. (2008). Salmon trypsin stimulates the expression of interleukin-8 via protease-activated receptor-2. *Toxicol Appl Pharmacol* 230 (3): pp 276 – 282

In our second paper we focus on salmon trypsin as an activator of inflammatory responses in airway epithelial cells *in vitro*. Unpublished results from our group have revealed that workers in the salmon industry are exposed to enzyme-containing aerosols generated during industrial processing of the fish. Based on the fact that tissue extracts from fish exhibit an inflammatory potential that can be linked to serine protease activity in addition to the knowledge that serine proteases like trypsin are highly active mediators with numerous biological functions, we wanted to investigate the capacity of salmon trypsin to initiate an inflammatory response in human airway epithelial cells. The stimulation of nuclear factor-kappa B (NF-κB) and interleukin (IL)-8 and the role of PAR_2 in this inflammatory signal mediation were investigated. Exposure of A549 cells to purified trypsin isolated from salmon (*Salmo salar*) yielded a concentration-dependent increase in the secretion of IL-8. By the use of siRNA to knock down the

endogenous expression of PAR_2 in human airway epithelial cells, we show that the increased secretion of IL-8 following trypsin stimulation was mediated by activation of PAR_2. Additionally, this paper demonstrates that PAR_2 activation by salmon trypsin is coupled to induction of NF-κB-mediated transcription in a PAR_2 transfected HeLa model. The results presented in this paper suggest that salmon trypsin, by activation of PAR_2, might influence upon inflammation processes in the airways if inhaled in sufficient amounts.

Paper III

Larsen AK, Kristiansen K, Seternes OM, Bang B. Molecular modelling of trypsin from the king crab (*Paralithodes camtschaticus*). *Manuscript – short communication.*

Molecular size, conformation and electrostatic potential are all factors that influence on a molecule's ability to bind and interact with signalling partners. Small chemical changes can inactivate the agonist-molecule, as the receptor may fail to bind the altered form or bind it less efficiently. The nature of the interaction between two signalling partners will influence upon downstream signalling pathways following molecular binding and receptor activation/inactivation. During purification of several trypsins from fish and king crab we observed that king crab trypsin bound stronger to the anionic column in the fast protein liquid chromatography (FPLC) compared to the other fish trypsins we purified. In paper III we show by molecular modelling techniques that king crab trypsin displays a more negatively charged surface compared to salmon and bovine trypsins. In addition, we identify 3 positions located near the substrate binding pocket at which a divergence in amino acid charge in the king crab trypsin molecule may influence upon the binding of substrate to PAR_2. The king crab trypsin is also shown to be a slightly bigger molecule compared to salmon and sardine trypsins. Despite the structural variation, no significant differences were found in enzymatic activity between bovine, salmon, sardine, and king crab trypsins measured by their ability to hydrolyzate a chromogenic substrate (DL-BAPNA). This paper presents preliminary results indicating that differences in electrostatic charge in residues at key interacting positions may result in altered binding capacity of the agonist in question, and that electrostatic interactions can be of importance in binding, cleavage and subsequent activation of PAR_2. Still, more extensive molecular modelling studies and cell based assays are necessary in order to identify essential residues in the king crab trypsin and how their interaction with binding residues in PAR_2 might influence upon receptor activation and possible functional selectivity.

Paper IV

Larsen AK, Seternes OM, Larsen M, Kishimura H, Rudeskaya G, Bang B. Purified sardine and king crab trypsin stimulate IL-8 secretion and NF-κB activation, at least partly, via PAR_2, but displays individual differences in transformation of the NF-κB-signal. *Manuscript*.

Respiratory symptoms are present in workers processing a great variety of seafood, including the salmon, sardine, and king crab industry. Knowing that salmon trypsin is able to induce secretion of IL-

8 from airway epithelial cells by activating PAR_2, we wished to explore if purified trypsins from king crab (*Paralithodes camtschaticus*) and sardine (*Sardinops melanostictus*) could induce similar effects in cell stimulation assays. In addition, research indicate that different types of seafood seem to display dissimilar irritant/allergic potencies and a higher prevalence of occupational airway symptoms is associated with exposure to aerosols from arthropods (crustaceans) than with bony fish and molluscs [6]. This knowledge inspired us to investigate if we could detect differences in intracellular signalling pathways coupled to IL-8 in human airway epithelial cells following stimulation with king crab and sardine trypsins. Paper IV shows that both sardine and king crab trypsin are able to induce secretion of the pro-inflammatory cytokine IL-8 from cultured human airway epithelial cells (A549) as previously shown for purified salmon trypsin. Both trypsins tested (sardine and king crab) generate DNA-binding of activated NF-κB, but displays individual differences in transformation of the NF-κB signal. High enzyme concentrations of king crab trypsin yields high levels of NF-κB that does not translate into increased secretion of IL-8 as opposed to salmon and sardine trypsins where the NF-κB levels correlate with the secreted amounts of IL-8 in all enzyme concentrations applied. However, siRNA results indicate that both the increased secretion of IL-8 and the generation of activated NF- κB following sardine and king crab trypsin stimulation is mediated, at lest partly, by activation of PAR_2.
In an attempt to reveal possible differences in the PAR_2 activating potential between the trypsins investigated, we further explored the contribution of MEK/ERK, p38 and NF-κB to the secretion of IL-8 following stimulation with purified sardine and king crab trypsins by the use of specific inhibitors.
The results from the inhibition assays indicate that MEK/ERK and NF-κB are both required for purified sardine and king crab trypsin-induced secretion of IL-8 but via separate pathways, while p38 may influence upon activation, nuclear translocation and/or DNA binding of activated NF-κB. Our inhibitor assays revealed no significant differences between purified sardine and king crab trypsin that might explain the observed discrepancy between NF-κB generation and subsequent IL-8 secretion in the upper concentration range of king crab trypsin
The differences in IL-8 and NF-κB dose-response relationships indicate that small structural variations in agonists may lead to differences in receptor activation and subsequent intracellular signalling. Future research focusing on possible biased signalling based on molecular structure, charge, and concentration are suggested.

Methodological considerations

Cell lines

In the presented work we wished to investigate if trypsins from seafood were able to induce inflammatory reactions in human airways by the use of techniques based on molecular biology. We chose to develop a majority of our assays using the A549 cell line. This line was initiated in 1972 through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male [315]. Further studies revealed that the cell line has properties of type II alveolar epithelial cells [316]. The A549 cell line is well known and widely used. Additionally it is shown to endogenously express PAR_2, the receptor of our interest [97]. Being an immortalized cell line it is fairly easy to work with and displayed good transfection efficiency. The latter of particular interest for us as we wanted to develop an assay based on receptor knockdown by the use of small inhibitory RNA (siRNA) to verify that our observed results in fact were attributable to PAR_2 activation. In the final half of this work we also utilized an A549 cell line stably transfected with a NF-κB-luc element giving us the chance to measure activated NF-κB directly without prior transfection of the NF-κB-luc reporter vector. This is an advantage as the protein expression in transiently transfected cells may vary more between assays. Our siRNA transfection protocol was prepared based on the wild type A549 cell line where a combination of three different pre-designed siRNAs targeting the PAR_2 gene were shown to reduce the expression of PAR_2 with 91% compared to cells receiving the negative control siRNA. However, follow up studies revealed that the A549NF-κB-luc cell line showed inferior transfection efficiency with a 54 – 72% reduction in PAR_2 expression when subjected to the same transfection protocol. This has to be kept in mind when comparing the results from paper II and the preliminary results in the manuscript of paper IV. The siRNA protocol for the A549NF-κB-luc+ strain is presently under optimization and the assays involving PAR_2 knockdown will be repeated before manuscript IV is submitted.
A great proportion of the knowledge regarding signal transduction originates from work involving immortalized cell lines. Even though the A549 cell line is frequently used in models investigating airway epithelial effects it has to be remembered that it is derived from a malignant tumor and therefore may possess differences in cellular signal transduction compared to physiologically normal cells.

The A549 cell line is derived from epithelial cells originating from the lower parts of the airways (alveolar epithelial cells). Because the observed airway symptoms in fish industry workers were from both upper and lower parts of the airways, we originally wanted to investigate if the trypsins were able to induce cytokine release from epithelial cells derived from the upper part of the airways as well. In order to confirm that the effects observed in the immortalized A549 cell line could be applicable to more physiological conditions, as far as can be expected using experiments involving cultured cells, we wanted this additional cell line to be of primary origin. We set out to use HNEPC, a primary human epithelial cell line derived from the nose, but by the use of real time PCR, this cell line were found to express only low amounts of endogenous PAR_2. Primary epithelial cell cultures derived from turbinate

mucosa is shown to express functionally active PAR_2 [317] while epithelial cells from nasal polyps are found not to express PAR_2 mRNA without prior stimulation [318]. Time limitations did not allow us to investigate the approach of establishing tissue sample derived cell cultures. Hence, the possible effects of seafood-derived trypsins on human primary nasal epithelial tissue are not included in this study.

The HeLa cell line (human epithelial cervical cancer cell line) was chosen for the assays involving DNA transfection and recombinant PAR_2 receptor expression because this cell line is previously shown not to express endogenous PAR_2 [1, 97, 319]. This allows us to isolate observed effects to PAR_2 as they will disappear in the wild type cells that are only transfected with control DNA. The use of HeLa cells were not chosen for the assays involving IL-8 measurements as they displayed a very high background level of IL-8 in non-stimulated cells.

Indicators of inflammation

Inflammation is a condition that involves numerous mediators and signalling pathways in different cell types and tissues. In the presented work we use some of these mediators as indicators of the potency to cause secretion of inflammatory mediators from cultured airway epithelial cells by the examined substances. Both the cytokine IL-8 and the transcription factor NF-κB are mediators that are closely linked to inflammation and they are additionally shown to be generated following PAR_2 activation [42, 178, 182]. IL-8 was chosen as an indicator of non-allergic inflammation in our cell stimulation assays. Although several cytokines may contribute to the initiation and progression of inflammation they were not included, as we merely chose IL-8 as an indicator and not set out to investigate the complete spectra of effects induced by seafood trypsins. We measured the secreted levels of IL-8 by ELISA as opposed to measurements of intracellular IL-8 levels or mRNA using other techniques, because the cytokines have to be secreted in order to exert their effects in promoting inflammation.
Additionally, we chose to investigate the possible connection between PAR_2 activation and DNA-binding of the transcription factor nuclear factor-κB (NF-κB). NF- κB transcription factors and the signalling pathways that activate them are central coordinators of innate and adaptive immune responses [320]. NF-κB is shown to be essential for induction of IL-8 transcription [186-189] and is additionally a transcription factor known to regulate a vast array of genes involved in inflammation [313, 314]. NF-κB was originally selected as an indicator of inflammation in addition to IL-8, giving us a stronger connection between the trypsins of interest and their ability to cause inflammation in cultured human airway cells. As the work progressed we observed differences in the trypsins with regards to their ability to activate NF-κB. Knowing that the transcription factors AP-1 and NF-IL6 are shown to be essential for maximal IL-8 expression it may have been of interest to investigate the influence of PAR_2 activation by trypsins upon the activation of these as well, but because of time limitations this will be for prospective studies.

Discussion

Occupational allergic reactions to seafood can among others manifest as rhinitis, conjunctivitis, and asthma [6], but few studies show a clear correlation between symptoms and increased levels of specific IgE towards fish. The lack of a strong IgE association implies that other mechanisms may be of importance in trigging the observed airway symptoms. Environmental proteases appear to be important in the pathogenesis of airway inflammation under certain conditions and their effects are to a large extent thought to be mediated by interactions with PARs. Exogenous serine proteases are shown to induce production and release of cytokines from airway epithelial cells, a process often shown to be mediated by PAR_2 activation. Some cytokines are contributing factors that amplify IgE-mediated inflammation [36], but environmental factors may also stimulate the production of cytokines in a non-IgE mediated process. Prior to this work, it has not been established that fish-derived serine proteases (trypsins) are capable of activating PAR_2 as reported for mammalian trypsins and other exogenous serine proteases [38, 39, 41, 47, 70, 73, 136, 137, 139, 141-144, 147, 148, 150].

IL-8 secretion by activation of PAR_2 by seafood trypsins

At the start of this work it was known that a wide range of proteases was able to induce production and release of IL-8 from airway cells [33-35, 37, 147, 148, 321, 322], an effect often shown to be mediated by activation of PAR_2. Knowing that PAR_2 is considered important for airway inflammation, we set out to explore if proteases from salmon could provoke secretion of IL-8 from A549 cells (human airway epithelial cells) and if purified salmon trypsin could activate PAR_2 as reported for mammalian trypsins.

In **paper I** we show that crude tissue extracts (pyloric caeca, stomach, skin mucus) from salmon are able to cause a concentration-dependent rise in IL-8 secretion from A549 cells. The majority of this increased secretion is believed to be caused by serine proteases as the use of serine protease inhibitors strongly attenuated the IL-8 levels. Other proteases may be involved as a protease inhibitor cocktail exhibited a more pronounced reduction of the IL-8 secretion compared to specific serine protease inhibitors alone. Fish skin/mucus and gastrointestinal tract are expected to contain Gram-negative bacteria as part of the normal bacterial flora. Lipopolysaccharides (LPS) from the cell wall of Gram-negative bacteria are shown to be a potent inducer of IL-8 production from human airway epithelial cells [161, 179, 323], an effect most probably mediated by interactions with Toll-like receptors [324]. The use of Polymyxin B (an inhibitor of LPS) did not influence upon the secreted IL-8 levels, giving us a strong indication that the increased IL-8 secretion was not due to endotoxins in the tissue extracts. The highest potency to cause increased IL-8 secretion were observed following stimulation with tissue extracts from pyloric caeca (part of the fish gut consisting of numerous blind sacs embedded in pancreatic tissue), a result to be expected as the intestines and particularly the pancreatic tissue contain large amounts of enzymes, with trypsin being the most important [325].

In **paper II** we confirm that purified salmon trypsin is able to induce secretion of IL-8 from human airway epithelial cells (A549) in a concentration-dependent manner. Secretion of IL-8 following trypsin stimulation in A549 is previously shown [42, 326]. Although these studies have demonstrated increased IL-8 secretion in response to stimulation with trypsin and PAR_2 agonist peptide, a firm correlation between cytokine secretion and trypsin-induced PAR_2 activation in this cell type has not been established. By the use of siRNA we were able to give direct evidence that the observed secretion of IL-8 following salmon trypsin stimulation is mediated by activation of PAR_2. The amount of secreted IL-8 was reduced with 70 – 85% in PAR_2 knockdown cells, leaving a slightly increased residue level in PAR_2 peptide agonist and salmon trypsin treated cells compared to untreated cells.
The fact that an observed effect (here; increased levels of IL-8) is abolished when the component expected to mediate the effect (here; PAR_2) is removed by siRNA is a strong indication of the components participation. So, if PAR_2 is responsible for the increased levels of IL-8 following purified salmon trypsin stimulation, why do we detect a slightly increased IL-8 level in the non-PAR_2 expressing A549 cells that have received siRNA to knock down the receptor? This may be due to the fact that siRNA treatment of the A549 cells resulted in a 91% knockdown of the receptor, still leaving 9% PAR_2 mRNA to be translated into functionally receptor protein. There might also be a possibility of purified salmon trypsin activating other signalling pathways leading to increased IL-8 secretion. Activation of PAR_1 and PAR_4 are, in addition to PAR_2, both shown to induce secretion of IL-8 in human airway epithelial cells [42]. Trypsin is previously shown to activate PAR_1 in addition to PAR_2, although in higher enzyme concentrations [63, 327] used in our cell based assays (40 nM versus 20 nM at the most in our assays). This makes us believe that the contribution of directly activated PAR_1 to the observed amounts of secreted IL-8 following sardine trypsin stimulation is minor, if at all present.
The PAR_2 agonist peptide, SLIGRL-NH_2, has been used as a positive control for PAR_2 mediated effects in most of our assays. As observed for the salmon trypsin, PAR_2 agonist peptide stimulation yielded a slightly increased IL-8 level in PAR_2 knockdown cells. This increased residue level could be due to unspecific effects of the agonist peptide yielding IL-8 secretion as well. The PAR_2 agonist peptide we used (SLIGRL-NH_2) is shown to be specific for PAR_2 [328], but PAR agonist peptides are functionally agonists at relatively high peptide concentrations (25 µM) and there is a possibility that these peptides might influence on receptors and signalling pathways besides PAR_2 [47]. These unspecific peptide effects can be controlled for by the use of inactive scrambled peptides. In our assays the inactive peptide LRGILS-NH_2 gave no secretion of IL-8, indicating that the increased residue level in PAR_2 knockdown cells following PAR_2 agonist peptide stimulation is not due to unspecific effects, but most likely is attributable to incomplete PAR_2 silencing.

In **paper IV** we corroborate that purified trypsins from sardine and king crab are able to induce increased IL-8 secretion from A549 cells as observed for crude salmon tissue extracts and purified salmon trypsin. The IL-8 secretion following stimulation with these trypsins is also concentration-dependent, but while purified sardine trypsin follows salmon trypsin with respect to the enzymatic concentrations yielding maximal IL-8 secretion, the purified trypsin from king crab stimulates maximal IL-8 secretion in a much lower concentration range. When measuring the enzymatic activity *in vitro*

using DL-BAPNA as a substrate there is no observed differences in the enzymatic activity of salmon, sardine, or king crab trypsin calculated as Units/µg protein that could explain this divergence.
Knockdown of PAR_2 reduced the secreted amount of IL-8 following stimulation with PAR_2 agonist peptide, sardine, and king crab trypsin, but not as pronounced as observed following salmon trypsin stimulation. We also observe that the reduction following PAR_2 agonist peptide stimulation is stronger (52%) than for the sardine and king crab stimulated cells (34% at the most). This in contrast to our finding in **paper II** where the reduction following PAR_2 agonist peptide and salmon trypsin stimulation in PAR_2 knockdown cells were nearly at the same level. There is a possibility that the purified sardine/ king crab trypsins activate other receptors leading to increased secretion of IL-8 in addition to the PAR_2 response, explaining the observed dissimilarity in agonist and trypsin-stimulated PAR_2 knockdown cells. However, the reduced decline in IL-8 secretion from PAR_2 knockdown cells after stimulation with sardine and king crab trypsins compared to salmon trypsin (34% at the most for sardine and 31% at the most for king crab versus 82% at the most for salmon trypsin) is most likely due to the A549NF-κB-luc+ cell type and its transfection capability compared to the wild-type A549 cell line used in paper II. Follow up studies with real-time PCR showed that siRNA targeting PAR_2 reduces the expression of the receptor mRNA levels with 54 – 72% in the A549NF-κB-luc+ cells as compared to 91% in the wild type A549 cell line. The probability for this scenario is strengthened by the fact that the level of IL-8 also declines less following PAR_2 agonist peptide stimulation in the A549NF-κB-luc+ cell type, from 85% reduced secretion in PAR_2 knockdown A549 wild type cells to 52% in A549NF-κB-luc+ cells.
Additionally, in paper II the results from PAR_2 knockdown cells were compared to non-transfected cells as initial investigative real-time PCR assays showed no reduction in PAR_2 expression following transfection with negative control siRNA. Subsequent assays conducted prior to paper IV showed a reduced secretion of IL-8 (26 – 30%) in mock-transfected cells receiving the negative control siRNA, a result probably due to unspecific cellular effects following the transfection routine. This resulted in our incorporation of the negative control siRNA in the assays that are described in paper IV, yielding minor reductions when comparing the PAR_2 knockdown cells to mock-transfected rather than non-transfected. Independent of comparisons made between the papers the level of IL-8 secreted from agonist treated PAR_2 knockdown A549NF-κB-luc+ cells was significantly reduced, showing that activation of PAR_2 provide a major contribution to the increased secretion of IL-8. Even though the results from the trypsin stimulated cells did not reach significance, the reduction observed in PAR_2 knockdown cells is most likely due to PAR_2 involvement. Besides rejecting a connection between compared groups/events, lack of significance can also be contributed to the number of observations included in the calculation. Smaller differences between groups would need a greater number of observations in each group in order to reach significance. As our results included 4 – 6 observations from at least 2 independent assays, the numbers from siRNA inhibition of PAR_2 following sardine and king crab trypsin stimulation may reach significance with a greater number of observations. However, the lack of significance in trypsin exposed cell is almost certainly due to incompletely knockdown of the receptor. To address the non-significant results, the siRNA transfection protocol for PAR_2 knockdown

in the A549NF-κB-luc cell line will be optimized and the related assays repeated before the manuscript is submitted.
Based on our results and the discussed facts, we are able to make the following conclusion;
Purified trypsins from different fish species and king crab induce increased secretion of IL-8 from human airway epithelial cells, an effect that is mediated by activation of PAR_2 following salmon trypsin stimulation, and at least partly by activation of PAR_2 following sardine and king crab trypsin stimulation.

Generation of NF-κB via activation of PAR_2

The transcriptional regulation of IL-8 expression in airway epithelial cells is complex and involves the transcription factors nuclear-factor kappa-B (NF-κB), NF-IL6, and activating protein (AP)-1 [301, 329, 330]. Binding of the NF-κB element is required for activation in all cell types studied, while the AP-1 and NF-IL6 sites are not essential for induction but are required for maximal IL-8 gene expression [186-189]. PAR_2 is shown to activate NF-κB as well as AP-1 and NF-IL6 in different cell types, including human alveolar epithelial cells (A549), emphasizing the importance of PAR_2 in inflammation [148, 178, 214, 331, 332]. Both trypsin and PAR_2 agonist peptide is shown to activate NF-κB [148, 178, 214, 331, 332], although few works have established a firm correlation between trypsin-induced PAR_2 activation and NF-κB binding in airway epithelial cells. This knowledge motivated us to examine whether purified salmon trypsin, by acting as a PAR_2 agonist, could activate NF-κB as reported for other serine proteases [178, 214, 331-335].

In **paper II** we established a firm correlation between PAR_2 activation by salmon trypsin and generation of NF-κB by the use of transiently transfected PAR_2+ HeLa cells, a cell type that is known to lack endogenous PAR_2 expression [1, 97, 319]. Purified salmon trypsin as well as PAR_2 agonist peptide was able to activate NF-κB in PAR_2 positive cells, while no response was detected in PAR_2 negative cells. In **paper IV** we utilized an A549 cell line stably transfected with a NF-κB-luc element giving us the chance to measure activated NF-κB directly without having to transiently transfect the cells prior to each assay. We show that both sardine and king crab trypsins are capable of activating NF-κB, an effect that seems to be at least partly PAR_2 mediated, as knockdown of the receptor using siRNA attenuates the NF-κB response. However, the remaining NF-κB levels in PAR_2 knockdown cells may be due to incomplete delivery of siRNA to the A549NF-κB-luc strain compared to the wild type A549 or activation of other receptors and pathways as already discussed for the IL-8 secretion.
According to the literature, NF-κB is reported to be essential for induction of IL-8 gene expression in several cell types/lines. We wished to explore whether this applied to the increased amounts of IL-8 secreted from A549 cells following sardine and king crab trypsins as well. By the use of an NF-κB inhibitor we confirm in **paper IV** that NF-κB is essential for IL-8 production from A549 cells following stimulation with PAR_2 agonist peptide and both sardine and king crab trypsins as the IL-8 secretion in inhibitor treated cells was completely abolished.
In conclusion, salmon trypsin induces DNA-binding of the NF-κB transcription factor via activation of PAR_2 in transfected cells. Trypsins from sardine and king crab induce DNA-binding of the NF-κB

transcription factor in human airway epithelial cells, an effect at least partly mediated by activation of PAR_2. The generation of NF-κB is essential for the increased secretion of IL-8 following stimulation with salmon, sardine, and king crab trypsins.

The activation of NF-κB by salmon, sardine, and king crab trypsins is dose-dependent rising up to a maximum level that plateaus at enzyme concentrations that detach the cells leaving them in suspension during the assay. All the trypsins tested seem to behave similar in capacity of activating NF-κB, but with regards to king crab trypsin we observed high levels of NF-κB at enzyme concentrations that yielded no detectable secretion of IL-8. For purified sardine the dose-response curve for IL-8 followed the generated levels of NF-κB, while for the purified king crab trypsin the NF-κB response in the higher enzyme concentrations generated strong levels of NF-κB that failed to translate into simultaneous IL-8 secretion. In fact, the IL-8 levels are reduced with 88 – 91% compared to the background levels in these tree high enzyme concentrations of king crab trypsin (0,4 – 1 – 2 mU). The fact that the concentration-effect curves for NF-κB follows the secreted amounts of IL-8 for the piscine trypsins, but not for the king crab trypsin puzzles us as the effects all seems to be mediated by PAR_2.

Some proteases are shown to cleave PARs at several sites, including activation and disabling sites, and the net result depends on the efficiency of cleavage at different locations [73, 122]. Differential cleavage is shown to be concentration-dependent with regards to tryptase and inactivation seems to occur at 100 nM, while activation is detected already at 1 nM of tryptase. The response produced by this dual ability to activate/inactivate PARs will depend on the concentration of agonist in the actual tissue. Theoretically, the king crab trypsin could be able to cleave PAR_2 at activation and disabling sites, and the net result of activation/inactivation may depend upon the concentration of enzyme. However, an inactivation of the receptor only explains the lack of IL-8 and not the increased amounts of NF-κB, presuming that both these observed effects are mediated by PAR_2 activation.
To investigate the ability of a given protease to cleave the N-terminal end of PAR_2, synthetic peptides corresponding to this segment may be used to determine the peptide fragmentation created by protease cleavage. The cleavage products can be examined by mass spectrometry to identify the sites of cleavage and by this give information whether the protease in question is capable of activating and/or inactivating PAR_2. However, although it has been demonstrated that N-terminal peptides of rat and human PAR_2 are rapidly cleaved by trypsin at multiple potential cleavage sites that would both activate and disarm the receptor [152] (Hollenberg MD, personal communication), rat PAR_2 expressed in intact cells displays selective tryptic cleavage only at the activation site without cleavage downstreams [336]. To assess activation/disarming of cell expressed human PAR_2, a Canadian research group have established a receptor desensitization assay [327]. By the consecutive application of trypsin, agonist peptide and/or the protease to be evaluated in varying orders, they assess if the receptor is activated or disarmed by the ability to cause intracellular calcium-signalling. Currently, cleavage data from synthetic peptides and activation data from the cell based receptor desensitization assay are generated for salmon, sardine, and king crab trypsins by our collaborators in Canada, Dr. Morley Hollenberg's group. We are eagerly awaiting their final results but preliminary studies indicate that

salmon, sardine and king crab trypsin are all capable of both activating and disarming human PAR_2 in the cell based receptor assay in addition to cleavage of synthetic peptides at activation and inactivation sites (Hollenberg MD – personal communication).

In theory, a scenario where the PAR_2 generated signal translating into NF-κB activation and IL-8 secretion are shut off due to receptor inactivation arising at this particular enzyme concentration of king crab trypsin, while activation of an unknown receptor generating the observed amounts of NF-κB due to a different signaling pathway where NF-κB results in transcription of different target genes besides IL-8, might be possible. NF-κB is a transcription factor known to regulate the expression of over 200 immune, growth, and inflammation genes [313] and are activated by a large number of receptors and pathways [314, 337].
Normally, IL-8 is barely secreted from non-induced cells, but increased production and secretion is rapidly induced by a wide range of inflammatory stimuli and cellular stress [186]. It is to be expected that the background level of IL-8 secreted in our assays is due to activation of different cellular receptors and intracellular signaling pathways depending on subtle fluctuations in the extra cellular environment during handling of cells grown *in vitro*, although we strive to standardize their conditions [303, 338, 339]. However, if this basal level theoretically could be due to PAR_2 involvement, an inactivation of the receptor might explain the reduced amounts of IL-8 compared to the background level observed in the higher enzyme concentrations (0,4 – 1 – 2 mU) of king crab trypsin.

There may also be a possibility that king crab trypsin cleaves the N-terminal end of PAR_2 differently compared to the fish trypsins and by this activating other PAR_2 coupled signalling pathways yielding different end products besides the secretion of IL-8. For many G protein-coupled receptors like those for angiotensin II, dopamine, serotonin, and adrenergic ligands, differential signaling depending on the activating ligand are now accepted [90-93]. This phenomenon is termed “agonist-biased signaling” or “functional selectivity”. Recent data also indicate that the proteolytically-revealed tethered ligand sequence(s) and the mode of its presentation to the receptor (tethered vs. soluble) can confer biased signaling by PAR_2 [89]. In the study by Ramachandran and co-workers activation of PAR_2 is measured by its ability to elevate intracellular Ca^{2+} and/or activate ERK/p42/44 MAP kinase in stably ratPAR_2-transfected KNRK cells, a cell type shown to express very low levels of functional PAR_2 [97]. The group shows that even though the ability of the tethered ligand to stimulate an elevation in intracellular Ca^{2+} is lost when the two first amino acids of the ligand ($S^{37}L^{38}$) is mutated to alanines or switched in order ($L^{37}S^{38}$), the activation of MAP kinase is retained. Furthermore, the synthetic PAR_2 activating peptide SLAAA-NH_2 activates MAP kinase, but is not capable of stimulating an elevation in intracellular Ca^{2+}. This in contrast to the scenario where the same sequence is presented to the receptor as a tethered ligand and in this form activates both MAP kinase and calcium signalling.
Along with the first two amino acids of the trypsin-revealed tethered ligand ($S^{37}L^{38}$), additional residues are considered important for receptor activation. Receptor chimera studies of the human-Xenopus PAR_1 receptor suggest a possible interaction between the positive Arg^5 in SFLL**R**N and the negative Glu^{260} in the second extracellular loop of the PAR_1 [85, 86]. These amino acids are also conserved in

PAR_2. Thus, an Arg^5-Glu^{260} interaction might also operate in recognition of the PAR_2 agonist peptide SLIG**R**L by PAR_2. Arg^5 in the PAR_2 agonist peptide SLIGRL corresponds to the positive arginine at position 41 of the revealed tethered ligand. When this residue is changed to either a neutral alanine or a negative glutamatic acid (changing SLIGRL to SLIG**A**L or SLIG**E**L) these sequences presented to the receptor in form of soluble peptides has a marked reduction in the ability to cause intracellular Ca^{2+} signalling (potency reduced by at least 32-fold for SLIGAL and 130-fold for SLIGEL). Interestingly, when Arg^{41} is changed in the tethered ligand the mutated receptors are able to give equivalent responses in intracellular calcium signalling showing that the trypsin-revealed PAR_2 tethered ligand sequence interacts differently for receptor activation that does the same peptide sequence as a free peptide [77]. Although these results diverge with respect to the response produced by mutated PAR_2 agonist peptides versus mutated tethered ligand, changes in electrostatic potential seems to be an important aspect in the binding of agonist to PAR_2 receptor.

During purification of several fish trypsins and trypsin from the king crab by fast protein liquid chromatography (FPLC), we observed that king crab trypsin bound stronger to the anionic column compared to the other fish trypsins. Knowing that molecular size, conformation and electrostatic potential will influence on a molecule's ability to bind and interact with signalling partners we decided to explore this divergence further.

In **paper III** we show by molecular modelling that the surface of king crab trypsin display a large area with strong negative electrostatic potential compared to the smaller negative areas of bovine and salmon trypsins. In addition these latter trypsins also display areas with strong positive electrostatic potentials, a feature lacking in king crab trypsin. Because of the lack of a full amino acid sequence of the sardine trypsin molecule we were not able to do any modelling of and comparison with sardine trypsin. The modelling of bovine, salmon, and king crab trypsins suggest that at least 3 divergent positions are located near the substrate binding pocket and might affect the binding of substrate to PAR_2.

1) The positive Arg^{244} in the king crab trypsin corresponds to the negative Glu^{221} in salmon trypsin and the neutral Gln^{199} in bovine trypsin.
2) The neutral Tyr^{247} in king crab trypsin corresponds to the neutral Asn^{224} in salmon trypsin and to the positive Lys^{202} in bovine trypsin.
3) The negative Asp^{196} in king crab trypsin corresponds to the neutral Met^{175} in salmon trypsin and the neutral Gln^{155} in bovine trypsin.

It is possible that the positive Arg^{36} (and/or positive Lys^{34}) of PAR_2 may interact differently with the binding pocket in the three trypsins. Because of differences in the electrostatic potential it is possible that PAR_2 might bind weaker to king crab trypsin than to other trypsins due to repulsive interactions between the positive Lys^{34}/ Arg^{36} in PAR_2 and the positively charged Arg^{244} in king crab trypsin. This residue corresponds to a negative amino acid (Glu^{221}) in salmon trypsin and a neutral amino acid (Gln^{199}) in bovine trypsin. Recently, Zhang and co-workers (2009) have documented that electrostatic

interactions presumably play an important role in alignment of the PAR_2 N-terminal end with its activating protease domain [340]. By the use of molecular simulations they show that positive amino acids in the proximity of the cleavage site of PAR_2 are located close to negatively charges residues on the binding pocket surface of the activating protease (factor VIIa [FVIIa]), whereas negatively charged residues from the N-terminal domain are close to positively charged FVIIa residues. These results indicate that electrostatic interactions in specific regions may guide substrate orientation.
Additionally, this report indicates that because of the large discrepancy in distance between the active site of FVIIa and the cell membrane compared to the cleavage site of PAR_2 and the cell membrane, the FVIIa molecule must be tilted towards the cells membrane in order to bind and cleave the PAR_2 extra cellular tail. This is a process that may need additional protein interactions. The king crab trypsin is shown to be a slightly bigger molecule (28 – 29 kDa) compared to salmon and sardine trypsins (23,7 – 25 kDa) [341-345]. Whether this slight increase in size would affect the ability of king crab trypsin to interact with the N-terminal domain of PAR_2 needs further investigation to be answered.

Although a weakened receptor interaction due to change in electrostatic potential might explain the differences between king crab and salmon trypsin with respect to NF-κB activation and IL-8 secretion, we would by this expect king crab trypsin to be a less potent PAR_2 agonist compared to salmon trypsin. According to pharmacological principles, an agonist with inferior affinity for a receptor require a higher concentration at the site of binding to produce a similar response compared to an agonist possessing superior affinity. Meaning that in order for the king crab trypsin to produce similar effect, here measured by the secretion of IL-8, comparable to sardine and salmon trypsins we would have to stimulate the cells with higher enzyme concentrations. Opposing to this we observed that king crab trypsin was able to induce secretion of IL-8 in a much lower concentration range than salmon and sardine trypsin as maximum response was seen with 0,02 mU of enzyme for king crab trypsin compared to 0,2 mU of enzyme for the sardine trypsin and 1 mU of enzyme for the salmon trypsin. However, the maximal level of IL-8 compared to untreated cells were lower in the king crab trypsin stimulations with a 9-fold increase, as opposed to a 18-fold increase in the sardine trypsin-stimulated cells and a 20-fold increase for the salmon trypsin. Regarding the ability to activate NF-κB both king crab trypsin and sardine trypsin produces maximum NF-κB response at the same concentration (0,4 mU of enzyme) but maximal level of NF-κB in king crab trypsin-stimulated cells yielded a 13-fold increase compared to untreated cells as opposed to a 9-fold increase for sardine trypsin (activation of NF-κB were not measured in the A549 cell type for salmon trypsin).
In brief summary, king crab trypsin seems to be more potent with regards to eliciting an IL-8 response, but less potent compared to the fish trypsins regarding the ability to produce maximal IL-8 secretion. Regarding the ability to generate DNA-binding of NF-κB, king crab trypsin seems to be more potent than sardine trypsin both with regards to induction of a response and the strength of the response. If these observed differences could be a result of the ability to activate or inactivate PAR_2 at different enzyme concentrations, due to agonist biased signalling yielding a differentiated response by activation of separate signalling pathways based upon the concentration of enzyme or originate from an inferior binding capacity due to electrostatic forces is presently unknown.

Knowing that binding of the AP-1 and NF-IL6 elements in addition to NF-κB are required for maximal IL-8 gene expression [186-189], it is tempting to speculate in the fact that these transcription factors may be regulated differently by the king crab trypsin compared to the fish trypsins. The measurements of NF-κB in this study were chosen in order to achieve a stronger connection to signaling pathways resulting in airway inflammation. Time limitations did not allow us to explore the relationship between the trypsins and the AP-1 and NF-IL6 binding sites, although this would be an interesting topic for prospective studies.

In conclusion, the generated NF-κB leads to increased secretion of IL-8 for all trypsins apart from the highest enzyme concentrations of king crab trypsin. The signaling events that lead to high levels of activated NF-κB without subsequent secretion of IL-8 in the elevated concentrations of king crab trypsin are presently unknown. However, based on the NF-κB readings from the siRNA inhibition assays, PAR_2 seems to be involved to some extent. Additionally, the mechanisms responsible for what, despite high NF-κB levels, seems to be an inhibition of the basal secretion of IL-8 in the highest enzyme concentrations of king crab trypsin needs further investigation to be explained.

Activation of MAP kinases as part of the PAR_2 signal transduction cascades leading to increased IL-8 secretion

Virtually all stressful and pro-inflammatory stimuli known to induce IL-8 production activate a number of protein kinases. Hoffmann and co-workers (2002) have determined that maximal IL-8 amounts are generated by a combination of three different mechanisms [186]:

1) De-repression of the gene promoter.
2) Transcriptional activation of the gene by transcription factors and c-Jun-N-terminal protein kinase (JNK) pathways.
3) Stabilization of the mRNA by the p38 mitogen-activated pathway.

In addition to NF-κB, activation of the MAP kinases ERK 1/2, JNK, and p38 is shown to be required for maximal IL-8 expression in TNFα-stimulated human airway epithelial cells [346]. The involvement of MAPKs in PAR_2-mediated IL-8 synthesis in airway cells is recently investigated and is found to be dependent on activation of ERK1/2 and JNK, while the involvement of p38 is more uncertain. ERK1/2 and JNK, but not p38 are reported to be involved in the PAR_2 agonist peptide induced IL-8 secretion from A549 cells in some studies [179, 181], while others report an involvement of all three kinases [178].

In **paper IV** we explored the contribution of MEK/ERK and p38 to the secretion of IL-8 following stimulation of A549-NF-κB-luc+ cells with purified sardine and king crab trypsins by the use of specific inhibitors. ERK 1/2 activation has been demonstrated to be required for IL-8 mRNA and protein expression in airway epithelial cells (A549) [347], and PAR_2 has been shown to activate ERK 1/2 in human bronchial epithelial cells (16HBE14o) [147] and the A549 cell line [179, 181]. In line with previous results we observed a pronounced reduction in the secreted levels of IL-8 when MEK/ERK was inhibited. The reduction was close to similar in PAR_2 agonist peptide (SLIGRL-NH_2), sardine trypsin, and king crab trypsin stimulated cells with 86, 84, and 65% respectively. The influence of inhibitors on

the NF-κB generation was also investigated to determine whether activation of the MAP kinases is essential for NF-κB activation or not. The NF-κB levels remained almost unchanged in the inhibitor treated PAR_2 agonist peptide and sardine trypsin stimulated cells, indicating that the contribution of MEK/ERK to the secretion of IL-8 is a NF-κB-independent process. However, in the king crab trypsin stimulated cells the NF-κB levels increased with 16 – 25% in inhibitor treated cells. Although not significant, the result could imply that king crab trypsin activates distinct signaling pathways that through MEK/ERK involvement contribute to inhibition of the generation, nuclear translocation and/or DNA-binding of activated NF-κB.

P38 is primarily believed to regulate the IL-8 protein expression at a post-transcriptional level stabilizing the IL-8 mRNA transcript [186, 346]. On the other hand, the p38 pathway is also shown to contribute to transcriptional activation by modulating the transactivation capacity of the NF-κB p65 subunit in TNF-activated murine fibrosarcoma cells [348]. P38 activity is also shown to be required to enhance the accessibility of the cryptic NF-κB binding sites contained in H3 phosphorylated promoters, indicating that p38-dependent H3 phosphorylation may mark promoters for increased NF-κB recruitment [349]. In primary human dendritic cells this p38-dependent phosphorylation and phosphoacetylation of histone H3 is shown to be induced by inflammatory stimuli, selectively occurring on the promoters of cytokine and chemokine genes. The involvement of p38 in PAR_2 mediated IL-8 secretion from human airway epithelial cells is presently uncertain. The use of a p38 inhibitor (SB203580) at 10 uM failed to affect the secretion of IL-8 in the study conducted by Ostrowska and Reiser (2008) [179], and Moriyuki and co-workers (2008) [181] observed no effect using the same inhibitor at 1 uM. Adam and co-workers (2006) [178] who reports of p38 involvement in the PAR_2 mediated secretion of IL-8 have also included inhibitor studies demonstrating inhibition of p38 using SB203580 at 25 uM.
We observed a pronounced reduction in the secreted levels of IL-8 following PAR_2 peptide (52%), sardine (35 – 45%), and king crab trypsin (40%) stimulation in A549 cells treated with p38 inhibitor (SB202190 at 10 uM). In contrast with former information we observed a reduced level of NF-κB following p38 inhibition both in peptide and trypsin stimulated cells (46% reduction in PAR_2 agonist peptide, 44 – 49% reduction in sardine trypsin, and 53% in king crab trypsin). This indicates that p38 in addition to regulating the IL-8 protein expression at a post-transcriptional level may influence upon activation of NF-κB, nuclear translocation or/and DNA binding of activated NF-κB in A549 cells.
Care should be taken when interpreting data obtained with inhibition of p38 MAPK by the pyridylimidazole analogues (SB203580, SB202190) and reflections should be made regarding the concentrations applied. These inhibitors are highly specific, but a few other kinases (Raf and lymphocyte kinase) are inhibited by SB203580, albeit less potently than p38 [350-352]. Hall-Jackson and co-workers (1999b) have shown that SB203580 inhibits c-Raf with an IC_{50} of 2 μM *in vitro*, however SB203580 at 10 – 25 μM does not suppress the classical MAP kinase cascade in mammalian cells (rat PC12 cells [353], human KB cells [353, 354], mouse C3H 10T1/2 cells [355], mouse Swiss 3T3 cells, or rat L6 myotubes [352]). On the contrary, SB203580 (10 μM) induces a remarkable activation of c-Raf that may counterbalance its inhibition [352]. These studies do not include SB202190, the inhibitor of our

choice, but SB203580 and SB202190 are structurally alike and similar effects may be expected. Activation of the protein kinase c-Raf triggers the sequential activation of MAPK kinase-1 (MEK1) and the MAP kinases ERK1/2. Inhibition of Raf would result in diminished activation of MEK/ERK and in our case a reduced secretion of IL-8, as activation of MEK/ERK previously are shown to be important for the observed IL-8 secretion in trypsin and agonist peptide stimulated A549 cells. Based on this information, it may be somewhat substantial to claim that the reduced amounts of secreted IL-8 in p38 inhibitor treated A549 cells must be due to inhibition of p38, however it is most likely.

With regards to the reduced level of NF-kB in p38-inhibitor treated cells, it is more unlikely that this could be due to an undesired inhibition of Raf and subsequent loss of MEK/ERK activation as we have shown that inhibition of this pathway does not influence upon the activation of NF-kB.

In order to confirm if the observed reduction in secreted IL-8 in fact is due to inhibition of p38 following the use of p38 inhibitors, blots indicating the presence of active (phosphorylated) and inactive (unphosphorylated) forms of p38 and ERK1/2 could be performed. Validation of the observed results could also be done by over-expressing a drug-resistant mutant of the protein kinase. If the observed effects do not disappear when introducing this drug-resistant mutant, they may be due to inhibition of other targets than the indicated kinase. Due to time limitations we were not able to perform these types of experiments for verification, although it would have been desirable. None of the addressed studies employing SB203580 for inhibition of p38 in the A549 cell type have confirmed their observed effects with additional studies [178, 179, 181]. Based on searches in available medical databases (PubMed, MedLine, EmBase etc.) we have not been able to identify any works using the p38 inhibitor of our choice (SB202190) in studying the contribution of p38 to increased secretion of IL-8 following PAR_2 activation.

In conclusion, increased secretion of IL-8 from human airway epithelial cells following stimulation with sardine and king crab trypsins are dependent on activation of the MAP kinases ERK 1/2 and p38, in addition to generation of the transcription factor NF-κB. The contribution of the ERK 1/2 pathway to the increased secretion of IL-8 is a NF-κB-independent process, while the contribution of the p38 pathway seems to influence upon activation, nuclear translocation and/or DNA binding of activated NF-κB in addition to a probable regulation of the IL-8 protein expression at a post-transcriptional level. Besides the slight, non-significant, increase in NF-κB following MEK/ERK inhibition in king crab trypsin stimulated cells, no obvious differences were found between the sardine and king crab trypsins that can explain the lack of IL-8 secretion despite high NF-κB levels in the elevated enzyme concentrations of king crab trypsin.

Summary

This work demonstrates that purified trypsins from salmon, sardine, and king crab induce increased secretion of IL-8 from human airway epithelial cells. The secretion is mediated by the activation of PAR_2 for salmon trypsin and most probable also for sardine and king crab trypsin although ineffective PAR_2 knockdown in A549NF-κB-luc cells prevents strong conclusions for the latter. As the siRNA protocol is currently optimized for this A549 cell strain, the PAR_2 knockdown assays will be repeated before paper IV is submitted. All trypsins activate the transcription factor NF-κB, a process that is mediated by the activation of PAR_2 for salmon trypsin and most likely for sardine and king crab trypsins as well.
Increased IL-8 secretion following sardine and king crab trypsin stimulation is dependent on the transcription factor NF-κB for induction and the MAP kinases ERK 1/2 and p38 for maximal expression. The involvement of the ERK 1/2 pathway to the increased secretion of IL-8 is a NF-κB-independent process, at least regarding the sardine trypsin. A moderate, but non-significant, rise in NF-κB levels following MEK/ERK inhibition during king crab trypsin stimulation could imply that king crab trypsin activates signalling pathways that through MEK/ERK involvement might inhibit the activation of NF-κB, but these results should only be treated as indicative. In contrast to previous research, we found the contribution of the p38 pathway to the increased IL-8 secretion to be partly connected to NF-κB activation, in addition to a probable regulation of the IL-8 protein expression at a post-transcriptional level as reported by others.

Different variants of trypsin are shown to cleave and activate PAR_2, but little research contains comparison of the downstream signalling response produced by different trypsins in human airway epithelial cells. At first glance the trypsins from fish and king crab seem to behave similarly in our cell-based assays. However, our research has revealed several areas where king crab trypsin differs from the fish trypsins:

1) The dose-response relationship for IL-8 in human airway epithelial cells shows that king crab trypsin is more potent in initiating secretion of IL-8 from human airway epithelial cells compared to salmon and sardine trypsin.
2) King crab trypsin yields a lower maximal level of secreted IL-8,
3) but a higher maximal level of activated NF-κB compared to the fish trypsins.
4) The generated NF-κB does not translate into subsequent IL-8 secretion in the highest enzyme concentrations as opposed to fish trypsin generated NF-κB.
5) King crab trypsin seems to inhibit the basal levels of IL-8 secretion in the highest enzyme concentrations.
6) King crab trypsin seems to activate signalling pathways that by the involvement of MEK/ERK exhibit negative regulation of NF-κB.

If these observed differences in the king crab trypsin are due to structural relationships in the king crab trypsin molecule influencing upon alignment of the PAR_2 N-terminal end with the protease and by this activating distinct signalling pathways, due to a concentration-dependent receptor activation-inactivation relationship, or if they are due to activation and involvement of other receptors are presently unknown.

A possible agonist biased signalling based on subtle differences in what has been expected to be similar agonists may answer many of the diverging results found following PAR_2 activation in different cell lines and tissues. Further investigation of this topic can contribute to a better understanding of PAR_2 related physiology and pathology.

Epidemiological considerations

Our results from cell based assays indicate that seafood trypsins have the potential to initiate inflammatory signalling in airway epithelial cells. In order to determine if exposure to trypsin could be the cause of observed airway symptoms in seafood industry workers, more extensive studies must be conducted. Methods for quantification of total protease activity in inhaled air samples at the workplace must be optimized, and studies addressing the effects of combined exposure should be executed as aerosols in the working environment are shown to contain additional components (molds, endotoxins, allergens) that may affect induction of airway inflammatory processes.

In order to speculate if inhalation of trypsin from fish or crustaceans may be a causative agent leading to airway inflammation and development of airway symptoms we need to consider the following;

- Is the amount of proteases found in the working environment sufficient to produce effects comparable to the observed results in the cell based assays?
- How and to what extent do the proteases found in environmental samples from the workplace come in contact with the airway tissue?

Measurement of enzymatic activity in water samples taken from work stations in the salmon industry show a protease activity that is well above the concentrations yielding maximal IL-8 secretion in the cell based assays (see fig 4). Samples collected in the king crab industry show somewhat lower trypsin-like activity compared to the environmental samples from the salmon industry. However, the concentration of king crab trypsin yielding maximal effect in the cell based assays is considerably lower than what we found for the salmon trypsin, leaving the levels measured in the environmental samples still 2 orders of magnitude above the maximal effective concentration in the cell based assays.

In order for the serine proteases detected in the environmental samples to induce airway symptoms they would have to come in contact with the airway tissue, an event that takes place if the proteases are inhaled. Many of the workstations in the salmon and king crab industry involve mechanical processing of biological material and the use of water or airjets creates small particles/ droplets harbouring biological material (bioaerosols) that may be inhaled. Measurements taken from the breathing zone of workers in the salmon industry show serine protease activity, measured by the running of gel zymography of filter extracts (Bang B. – unpublished results). The protease activity of these filter extracts are too low to be measured by the serine protease assay described above, and optimization of methods to be used for quantification of proteases in air samples must be conducted before we are able to assess the amount of inhaled proteases. It has to be mentioned that the amount of trypsin applied in the cell based assays would be equally difficult to measure as the concentration of the stimulation solutions are based upon serial dilutions of the purified enzyme originally measured by the serine protease assay.

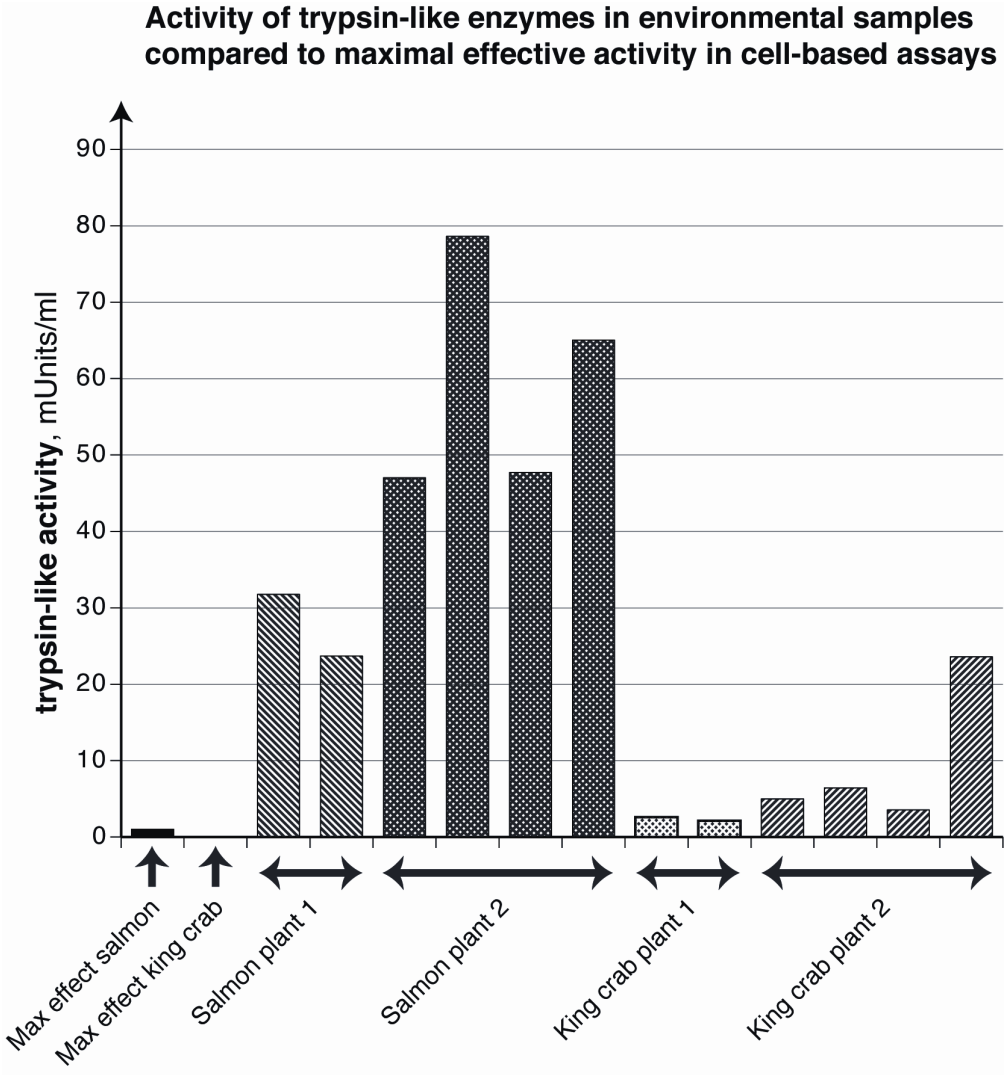

Figure 4

The trypsin-like activity in water sampled at workplaces in the salmon and king crab industry was measured in a serine protease assay where the hydrolyzation of a chromogenic substrate (DL-BAPNA) was determined just as described for purified trypsins [1] and expressed as milliunits (mU)/ml (Bang B. – unpublished results). Max effect for king crab trypsin in the cell stimulation assays (0,02 mU) is too low to be depicted in the diagram.

Based on the results we have from environmental samples collected in the fish industry, it is not unlikely that fish and crab industry workers may inhale proteases in amounts that could affect the airway epithelial cells. Purified trypsins from fish and king crab are shown to induce production and secretion of inflammatory mediators (NF-kB, IL-8) in our cell based assays, but whether inhalation of trypsins is the sole cause of the observed symptoms or if they contribute to a situation with combined exposure (proteases, molds, endotoxin, allergens, cold environment) creating potentiating or related effects needs further investigation to be answered.

Future perspectives

The signal transduction involved in airway inflammation caused by serine proteases encompasses numerous mediators most probably originating from a complex interplay between different receptors. Prospective research should seek to identify the mutual roles of the components implicated and elucidate the contribution made from PARs to the inflammation response. The results presented in this work strongly indicate that trypsins originating from fish and king crab have the potency of initiating or contributing to inflammatory reactions in human airway epithelial cells through the activation of PAR_2. Although we have chosen to focus on PAR_2 and its possible role in mediating inflammatory signals, other PAR-family members may also be involved in a tissue response toward serine proteases. Preliminary results from our collaborators in Canada, Dr. Morley Hollenberg and co-workers, show that salmon, sardine, and king crab trypsins are all able to cleave and unmask the tethered ligand sequence of short synthetic peptides corresponding to the N-terminal end of PAR_1 and PAR_4 in addition to PAR_2. Additionally, salmon trypsin is shown to activate calcium signalling via human PARs $_1$ and $_2$ in human embryonic kidney cells (HEK) and both salmon, sardine, and king crab trypsins are shown to aggregate rat platelets via rat PAR_4 (Hollenberg M.D. – personal communication). Trypsin is also previously shown to activate PAR_1 in addition to PAR_2, although in higher enzyme concentrations than the ones used in our cell based assays [63, 327].

PAR-signalling in intact tissue is most likely a multifaceted interplay between different PAR-family members. Knowing that **inter**molecular receptor activation between different PAR family members, in addition to the most common picture described with **intra**molecular receptor activation by the receptors own tethered ligand, is a probable scenario, the contribution of other PAR family members to the inflammatory signal initiated by PAR activation following trypsin stimulation in human airway epithelial cells would be highly interesting to investigate. Airway epithelial cells are shown to express both PAR_1 and PAR_2 [73, 109] and their expression relative to each other in the A549 cell line could be investigated by the use of real time PCR and immunohistochemistry. Furthermore, knockdown of the receptors, both individually and combined, by the use of siRNA could be used to detect their relative contribution to the release of inflammatory mediators.

Agonist biased signalling by PAR_2 has recently received attention and might be one explanation for the observed discrepancy in effects following receptor activation in different cell lines and tissues. Knowing that the king crab trypsin molecule displays a different net molecular charge and harbours substituted amino acid residues at seemingly important interaction points close to the substrate binding pocket, conducting more extensive molecular modelling studies would be of interest in order to explore the impact this would have on receptor binding, cleavage and activation. The results presented by us indicate that king crab trypsin may behave differently in activating PAR_2 compared to trypsins from fish, as shown by the high levels of activated NF-κB, at least partly mediated by PAR_2, which fails to translate into subsequent IL-8 secretion in the higher enzyme concentrations. Studies using PAR_2 receptor mutants as well as trypsin mutants would be effective in generating information about important residues for receptor cleavage and activation. This might be used in the attempt of

identifying possible interaction points where the enzyme-receptor binding differ between king crab and fish trypsins, perhaps giving rise to biased signalling. In order to understand the complexity of trypsin-induced PAR signalling it is important to determine the extent of biased signalling that might arise following receptor activation with what previously were considered to be agonists with similar effects. Knowing that the transcription factors AP-1 and NF-IL-6 are shown to be necessary for maximal expression of IL-8, their relative contribution to the IL-8 secretion following stimulation with king crab and fish trypsins would be interesting to determine. One hypothesis might be that biased signalling following PAR_2 activation by king crab trypsin leads to differential activation of the transcription factors NF-κB, AP-1, and NF-IL-6 according to enzyme concentration.

Future projects should also aim to link our previous results closer to the real situation by taking into consideration the mixed nature of biological exposures in work environments. The concomitant exposure of enzymes, allergens, micro organisms, and microbial components will cause activation of several signalling pathways at the same time. The activation of one signal pathway may thus exacerbate or attenuate the signal from another pathway. Microbial derived endotoxins, peptidoglycans, and β(1-3)-glucan are all signalling though surface receptors coupled to intracellular pathways with a number of possible cross-communication links with the PAR-mediated signals. LPS is shown to up regulate the expression of PAR_2 in human airway epithelial cells and combined stimulation with LPS and PAR_2 agonist peptide have a potentiating effect on the stimulation of the IL-8 synthesis [161]. Samples taken from the breathing zone of workers in the seafood industry shows that inhalation of aerosols containing endotoxins and micro organisms in addition to compounds with different enzymatic activity is possible [2]. Furthermore, the proallergic cytokine, thymic stromal lymphopoietin (TSLP), secreted by airway epithelial cells represent a cross-communication link between the innate inflammatory and the allergic (Th-2) cytokine cascades [356]. Trypsin is shown to induce production of TSLP from airway epithelial cells through PAR_2 [270].
Knowing that concomitant stimulation with different agents can initiate an intricate interplay in receptor expression and activation, combinatorial effects of different exposures in airway cells should be investigated in order to approach the possible pathogenesis of the observed airway effects in fish industry workers. Considered that PAR_2 activation is shown to involve both innate and acquired immune responses, studies with the aim of identifying the mutual contribution of these responses to the effects observed following PAR_2 activation *in vitro* and *in vivo* will be important for an improved understanding of the role of PARs in physiology and pathology.

Abbreviations

A549 cells	Human pulmonary epithelial cell line
AP-1	Activator protein 1
BAL	Bronchoalveolar lavage
BEAS-2B cells	Human bronchial epithelial cell line
C3H 10T1/2 cells	Mouse embryo-derived cell line
cAMP	Cyclic adenosine monophosphate
c-Cbl	E3 ligase
CD11/CD18	Integrins, leukocyte adhesion molecules
CG	Cathepsin G
c-Jun	Component of the AP-1 transcription factor complex
COPD	Chronic obstructive pulmonary disease
COS-7 cells	Simian kidney cell line
COX-2	Cyclooxygenase-2
c-Raf	MAP kinase kinase kinase (MAP3K)
CREB	cAMP responsive element binding protein
DAG	Diacylglycerol
Der P3/Der P9	Serine proteases from *Dermatophagoides pteronyssinus* and *D. farinae* (dust mite)
DL-BAPNA	Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride
DUB	Deubiquitinating protease
EBC-1 cells	Human pulmonary squamous cell carcinoma cell line
EC_{50}	Median effective concentration (required to induce a 50% effect)
ECL	Extra cellular loop of PAR
EGF-R	Epidermal growth factor receptor
EPa	Elastolytic metalloprotease from *Pseudomoas aeruginosa* (LasB or pseudolysin)
ERK	Extracellular-signal regulated kinase
FAK	Focal adhesion kinase
FVIIa	Coagulation factor IIa
FXa	Coagulation factor Xa
GDP	Guanosine diphosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GRK	G protein receptor kinases
GTP	Guanosine triphosphate
HAT	Human airway trypsin-like protease
HBE cells	Human bronchial epithelial cell line
HEK293 cells	Human embryonic kidney cells
HeLa cells	Human epithelial cervical cancer cell line
HNEPC	Human nasal epithelial cell line
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
IC_{50}	Half maximal inhibitory concentration (required to induce a 50% inhibition)
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IgE	Immunoglobulin E
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
IP3	Inositol-1,4,5-trisphosphate
Jab1	Jun activating binding protein-1
JNK	C-Jun-N-terminal kinase/stress-activated protein kinase (SAPK), class of mitogen-activated protein kinase
Jun D	Component of the AP-1 transcription factor complex
KB cells	Human epidermoid carcinoma cell line
KLKs	Kallikreins
KNRK cells	Rat kidney fibroblast cell line
LepA	Large exoprotease with a trypsin-like serine protease motif from *Pseudomonas aeruginosa*
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCT	Mast cell tryptase
MEK	MAPK/ERK kinase
MIP-2	Macrophage inflammatory protein 2
MMP-9	Matrix metalloprotease 9
MT-SP1	Membrane-type serine protease 1

NE	Neutrophil elastase
Neu7 cells	Rat primary cortical astrocyte cell line
NF-IL6	Nuclear factor for interleukin-6 (C/EBP; CCAAT/enhancer binding protein beta)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OA	Occupational asthma
OR	Occupational rhinitis
P22	Tryptic-like serine protease from rat brain
P38	Class of mitogen-activated protein kinase
PAR	Protease activated receptor
PAR-AP	PAR activating peptide/PAR agonist peptide
PC-3 cells	Human prostate cancer cell line
PC-12 cells	Rat adrenal medulla pheochromocytoma cell line
Pen c 13	Serine protease from *Penicillium citrinum*
PGE_2	Protaglandin E_2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLCβ	Phospholipase Cβ
PR3	Proteinase 3
RgpB	Arginine-specific cysteine protease from *Porphyromonas gingivalis*
Rho A	Ras homolog gene family, member A
RhoGEF	Rho-activating guanine-nucleotide exchange factors
ROCK	Rho-associated kinase
SAEC	Small airway epithelial cells
SiRNA	Small interfering/inhibitory ribonucleic acid
SW620	Human colon carsinoma cell line
Swiss 3T3 cells	Mouse embryo-derived fibroblast like cell line
TF	Tissue factor
TL	Tethered ligand
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
16HBE cells	Human bronchial epithelial cell line

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