Paper IV

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.

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Abstract

Respiratory symptoms are present in workers processing a great variety of seafood, including the salmon, sardine, and king crab industry. We have previously shown that salmon trypsin is able to generate DNA-binding of NF-κB and induce secretion of IL-8 from airway epithelial cells by activating PAR-2. In this study we explore if purified trypsins from king crab (*Paralithodes camtschaticus*) and sardine (*Sardinops melanostictus*) is able to induce similar effects in cell stimulation assays. Different types of seafood seem to display dissimilar irritant/allergic potencies in human airways and molecular modelling has identified divergent positions in the king crab trypsin compared to salmon trypsin that might influence upon the binding to the N-terminal end of PAR-2, which is a prerequisite for proteolytic activation of the receptor. This knowledge inspired us to investigate if we could detect differences in intracellular signalling pathways coupled to IL-8 in human airway epithelial cells (A549) following stimulation with purified king crab and sardine trypsin. Both sardine and king crab trypsin induce secretion of IL-8 from human airway epithelial cells in a concentration-dependent manner and generate DNA-binding of activated NF-κB. By the use of siRNA we can conclude that these effects are both mediated, at lest partly, through the activation of PAR-2. The king crab and sardine trypsin displays individual differences in transformation of the NF-κB signal, as high enzyme concentrations of king crab trypsin yields high levels of NF-κB that does not translate into increased secretion of IL-8 in the cell stimulation assays. The contribution of MEK/ERK, p38 and NF-κB to the secretion of IL-8 following stimulation with purified sardine and king crab trypsins were explored by the use of specific inhibitors. The results demonstrate 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. P38 was also found to contribute to the secretion of IL-8 by seemingly NF-kB-dependent processes.
The data presented indicate that small structural variations in agonists may lead to differences in receptor activation and subsequent intracellular signalling.

Introduction

The amount of evidence that connects proteases to airway inflammation is increasing. Secretion of cytokines from the airway epithelium contributes to the inflammation response. Cell stimulation assays show that increased cytokine secretion can be induced by both endogenous proteases from the coagulation cascade and inflammatory cells (Schoenmakers *et al*., 2005; Wang *et al*., 2006), as well as exogenous proteases derived from mites (King *et al*., 1998; Tomee *et al*., 1998), molds (Borger *et al*., 1999; Kauffmann *et al*., 2000), bacteria (Lourbakos *et al*., 2001; Ubl *et al*., 2002; Kida *et al*., 2007; 2008), cockroaches (Bhat *et al*., 2003), and fish (Larsen *et al.,* 2008). In addition to their well known role in digestion of dietary proteins, blood coagulation, and homeostasis, recent studies have revealed a novel role of serine proteases as signalling molecules acting via protease-activated receptors (PARs) in innate and adaptive immunity (Shpacovitch *et al*., 2008). PARs have emerged as important receptors in airway inflammation and allergy, and PARs are expressed in all cell types that participate in the inflammatory response of the lung; epithelial cells, mast cells, macrophages, infiltrated neutrophils and eosinophils, fibroblasts, smooth muscle cells, endothelial cells, lymphocytes, and neurons (Cocks *et al*., 1999; Ramachandran and Hollenberg, 2008).

Receptor activation by proteases is achieved by proteolytic cleavage of the N-terminal sequence, which unmasks a new amino terminus that serves as a tethered ligand that binds to conserved regions in the body of the receptor, resulting in the initiation of signal transduction (Ossovskaya and Bunnett, 2004). Exogenously applied synthetic peptides based on the sequence of the tethered ligand are also capable of activating PARs by directly binding to the body of the receptor. To date, four PARs have been cloned and characterized; PAR-1, PAR-2, PAR-3, and PAR-4. In contrast to the remaining PAR family members, PAR-2 is resistant to thrombin, but can prototypically be activated by trypsin (Nystedt *et al*., 1995; Böhm *et al*., 1996; Alm *et al*., 2000; Cottrell *et al*., 2004). A number of other proteases like mast cell tryptase (Molino *et al*., 1997), tissue factor (TF)-factor VIIa/factor Xa complex (Camerer *et al*., 2000), membrane-type serine protease-1 (MT-SP1) (Takeuchi *et al*., 2000), dust mite allergens Der P3 and Der P9 (Sun *et al*., 2001), mold allergen Pen c 13 (Chiu *et al*., 2007), and bacterial proteases like R-gingipain-B (Lourbakos *et al*., 2001), thermolysin (Ubl *et al*., 2002), serralysin (Kida *et al*., 2007), and LepA (large exoprotease) (Kida *et al*., 2008) are also shown to activate PAR-2 in experimental settings. Cleavage and activation of PAR-2 by different proteases might vary according to the type of cell that express the receptor, and is also shown to depend on the glycosylation status of the receptor (Compton *et al*., 2001; 2002).

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 signalling by PAR-2, its arrestin recruitment, and its internalization (Ramachandran *et al*., 2009). Thus, PAR-2 can signal to multiple pathways that are differentially triggered by distinct protease-revealed tethered ligands and soluble peptide agonists. For many other G-protein-coupled receptors, such as those for angiotensin II, dopamine, serotonin, and adrenergic ligands, differential signalling depending on the activating ligand (termed “agonist-biased signalling” or “functional selectivity”) are now accepted (Wei *et al*., 2003; Galandrin *et al*., 2007; Kenakin, 2007; Urban *et al*., 2007).

PAR-2, like other PARs, couples to $G_{q/11}$ protein that mediates downstream signal transduction upon receptor activation (Ossovskaya and Bunnett, 2004). Besides the activation of phospholipase Cβ, formation of inositol triphosphate and diacylglycerol, the receptor is shown to trigger the activation of other downstream signalling pathways including the mitogen-activated protein kinase (MAPK) cascades in distinct cell types. Additionally, studies indicate that maximal interleukin (IL)-8 protein expression requires activation of the MAP kinases ERK, JNK, and p38 as well as activation of the transcription factor NF-κB (Blackwell and Christman, 1997; Gon *et al*., 1998; Matsumoto *et al*., 1998; Hashimoto *et al*., 1999; Chen *et al*., 2000; Li *et al*., 2002).

Inhalation of particulate matter may affect the airway epithelial cells and previous studies have shown that production workers in the seafood industry inhale aerosols containing biological material (Bang *et al*., 2005; Jeebhay *et al*., 2005). Reports by Bang and co-workers (2005) and Shiryaeva and co-workers (2010) show an enhanced occurrence of respiratory symptoms from both the upper and lower parts of the airways in workers in the Norwegian salmon industry. The majority of the observed symptoms could not be explained by IgE-mediated processes as the prevalence of specific IgE was very low (none of the examined workers in the study conducted by Bang and co-workers (2005) 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)). Recently, we have shown that purified salmon trypsin induces secretion of the inflammatory mediator IL-8 and generate DNA-binding of NF-κB via activation of protease-activated receptor (PAR)-2 (Larsen *et al*., 2008).

Allergic diseases associated with occupational exposure to crab are well characterized (Jeebhay *et al*., 2001). Several studies report a high incident of occupational asthma in workers processing snow crab (*Chionoecetes opilio*) and king crab (*Paralithodes camtschaticus*), but other symptoms as rhinitis, conjunctivitis, and skin rash are also registered (Orford and Wilson, 1985; Cartier *et al*., 1986; Ortega *et al*., 2001; Howse *et al*., 2006; Gautrin *et al*., 2009). Although a majority of the literature point to an IgE-mediated mechanism for the development of respiratory disease among crab processing workers, there are reports that fail to show a clear correlation between crab specific IgE and new incidents of asthma-like and bronchitis cases (Ortega *et al*., 2001). Workers processing saltwater bony fish (sardine (*Sardinops sagax*) and anchovy (*Engraulis capensis*)) also appear to be at increased risk for developing work-related upper and lower allergic respiratory outcomes (Jeebhay *et al*., 2008). Like many of the studies conducted in the fish industry, the prevalence of work-related symptoms in the Jeebhay-study was higher than diagnosed occupational disease due to fish and allergic sensitization was reported only for a minority.

Knowing that purified 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. Additionally, different types of seafood seem to display dissimilar allergenic/irritant potencies and

higher prevalence of occupational allergic outcomes/airway symptoms is associated with exposure to aerosols from arthropods (crustaceans) than with pisces (bony fish) and molluscs (Jeebhay *et al*., 2001). Based on this knowledge, we wished 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.

Matherials and methods

Materials

Selective PAR-2 peptide agonist, Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-NH_2), the inactive control peptide, Leu-Arg-Gly-Ile-Leu-Ser-amide (LRGILS-NH_2), Na-Benzoyl-$_{D,L}$-arginine 4-nitroanilide hydrochloride (DL-BAPNA), and DMSO (D-2650) were purchased from Sigma-Aldrich, MO, USA. P38 inhibitor (SB203580) was purchased from Alexis Biochemicals, Lausen, Switzerland, while MEK inhibitor (U0126) was purchased from Promega, WI, USA and NF-κB inhibitor (BAY 11-7082) was purchased from Calbiochem, Merck4Biosciences, Darmstadt, Germany. Purified king crab trypsin was a kind gift from Dr. Galina N. Rudenskaya (Moscow State University), and the purified sardine trypsin was kindly supplemented by Dr. Hideki Kishimura (Hokkaido University).

Cell culture

A549/NF-κB-luc cells, a human pulmonary epithelial cell line stable transfected with a NF-κB binding luciferase reporter construct (Panomics P/N LR0051), were purchased from Panomics (no. RC0002), CA, USA, and were cultured in Dulbecco's minimum essential medium/Ham's F12 medium (1:1) (Gibco, NY, USA) supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine (Invitrogen), 50 IU/ml penicillin, 50 µg/ml streptomycin (Invitrogen), and 100 µg/ml hygromycin B (Sigma-Aldrich). The cells were passed without the use of trypsin by using a non-enzymatic cell dissociation solution (Sigma-Aldrich).

Preparation of crude enzyme

The preparation of sardine and king crab trypsin was conducted by our collaborators as described by Kishimura *et al*. 2006, Rudenskaya *et al*., 2000, and Kislitsyn *et al*., 2003.

Fast protein liquid chromatography (FPLC)

In order to standardize the purification processes, both trypsins were purified by fast protein liquid chromatography. All purification steps were carried out at 0-4℃. The freeze dried trypsins were re-suspended in 25 mM TrisHCl, pH: 7.5 and applied to a 1,5 ml Benzamidine-Sepharose 6B column equilibrated with 25 mM TrisHCl pH: 7.5, 10 mM $CaCl_2$, 500 mM NaCl. Bound trypsin were eluted using 120 mM Benzamidine and collected in 1,5 ml fractions. All fractions with enzymatic activity measured by the serine protease assay (DL-BAPNA) were pooled and dialysed against 25 mM TrisHCl pH: 7.5, 10 mM $CaCl_2$ at 4℃ over night using 10K Slide-A-Lyzer dialysis cassettes from Pierce, IL, USA. The following day the benzamidine purified trypsins were applied to a 1 ml Resource Q ion exchange column equilibrated with 25 mM TrisHCl pH: 7.5, 10 mM $CaCl_2$ and the enzymes were eluted with 1 M NaCl using a 7,5% gradient for 10 fractions (total of 5 ml) followed by a linear gradient rising to 100% in 20 fractions (total of 10 ml). Fractions corresponding to the observed peaks were tested for enzymatic activity and pooled before dialysis as described previously.

Protease activity determination

The enzymatic activity of the purified trypsins was determined by a serine protease assay where the hydrolyzation of a chromogenic substrate (DL-BAPNA) was measured spectrophotochemically by the increase in absorbance at 405 nm at room temperature for the length of 10 min. The substrate was diluted in substrate buffer (25 mM Tris/HCl, 10 mM $CaCl_2$, 2% (v/v) DMSO, pH 8.1) and used at a final concentration of 0,5 mM. The activity is measured in a total volume of 250 ul (10 ul of enzyme and 240 ul of diluted substrate) in clear, 96 well trays with flat bottom (BD Falcon, NJ, USA). The results were expressed as units (U)/ml (Outzen *et al*., 1996), and one unit of activity was defined as 1 µmol substrate hydrolyzed per minute using an extinction coefficient of 8800 $M^{-1}cm^{-1}$ (Erlanger *et al*., 1961). The calculations were made using the following formula:

$$\text{Unit: } \frac{dA}{dt} \times \frac{1}{(\varepsilon \times \text{optical path lenght}) \times 10^6} \times V_{final}$$

dA/dt = rate of absorbance change
ε = extinction coefficient

Cell stimulation assays

A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded in 6 well plates (9,6 cm^2) and were cultured until 80-90% confluence was reached, before the cells were starved for serum over night. Cells were exposed for different concentrations of purified trypsins, PAR-2 peptide agonist, or inactive control peptide for the appropriate time. In the inhibition assays the cells were pre-incubated with 10 µM (SB203580, U0126) or 100 µM (BAY 11-7082) of inhibitors for 1 hour at 37 C prior to the execution of the assay. Heat-inactivation was conducted by incubating the trypsins at 95°C for 15 min. Culture supernatants were harvested and kept at -20°C until the level of IL-8 was measured.

Measurement of IL-8 secretion

The amount of secreted IL-8 in the supernatant was determined by using an ELISA (enzyme-linked immunosorbent assay)-kit from BD Biosciences, NJ, USA, according to the manufacturer's protocol. The absorbance was read using an iEMS Multiscan EX (Thermo Labsystems). IL-8 production was expressed as pg/ml supernatant.

Small interfering RNA

A mix of three pre-designed siRNAs (Ambion, USA, catalogue nr 16704, ID nr 1960, 1876, and 1783) for the PAR-2 gene was used. The sequences of the siRNA primers were as follows: nr 1960; forward primer 5'-GGAGUUACAGUUGAAACAGTT-3', and reverse primer 5'-CUGUUUCAACUGUAACUCC-TT-3', nr 1876; forward primer 5'-GGAAGAAGCCUUAUUGGUATT-3', and reverse primer 5'-UACCA-AUAAGGCUUCUUCCTT-3', and nr 1783; forward primer 5'-GGAACCAAUAGAUCCUCUATT-3', and reverse primer 5'-UAGAGGAUCUAUUGGUUCCTT-3'. The Silencer negative control nr 1 (Ambion, catalogue nr 4611) was used as a control siRNA. The primer sequences of the negative control were as follows: forward primer 5'-AGUACUGCUUACGAUACGGTT-3', and reverse primer 5'-CCGUAUC-GUAAGCAGUACUTT-3'. A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded in 6 well plates (9,6

cm^2) and were transfected at 70-80% confluency using Lipofectamine 2000 (Invitrogen)-assisted transfection according to the manufacturer's protocol.

Reporter gene assay

A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded in 6 well plates (9,6 cm^2) and were cultured until 80-90% confluence was reached, before the cells were starved for serum over night. Cells were exposed to different concentrations of purified trypsins, PAR-2 peptide agonist, or inactive control peptide for the appropriate time as in the cell stimulation assays. The cells were lysed using lysis buffer (Applied Biosystems, CA, USA) and the luciferase activity was determined on a Luminoscan Ascent (Thermo Electron Corporation, Vantaa, Finland) using the Dual-Light Luciferase Reporter gene Assay System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The luciferase activity was normalized with respect to protein concentration in the lysates by the use of a DC Protein Assay (Bio-Rad, CA, USA).

Statistical analysis

Group data were compared using Student's *t* test for independent samples (SPSS). Differences were considered significant for *p* values of < 0.05.

Results

Purified king crab trypsin induces secretion of IL-8 in A549/NF-κB-luc cells in a different concentration range than fish trypsins

We have previously shown that salmon trypsin can induce secretion of the pro-inflammatory cytokine IL-8 in human airway epithelial cells (Larsen *et al.,* 2008). In order to determine whether trypsin from other species used in the seafood industry can enhance the secretion of IL-8 in human airway epithelial cells, A549/NF-κB-luc cells were stimulated with different concentrations of purified king crab and sardine trypsin for 6 h. The results depicted in figure 1a show that purified sardine trypsin as well as purified king crab trypsin caused an increase in the secretion of IL-8 in a concentration-dependent manner, but the concentration range that lead to IL-8 secretion were different for the two trypsins. Maximum response was seen with 0,2 mU of sardine trypsin with a 18 fold increase compared to untreated cells, while the maximum peak for king crab trypsin was reached already at 0,02 mU with a 9 fold increase above untreated cells.
With regards to the sardine trypsin, the level of IL-8 decreased with high enzyme concentrations until barely detectable in the concentrations that also lead to detachment of the cells (1 mU – 2 mU). The king crab trypsin behaved somewhat different as we observed that the level of IL-8 fell already at lower concentrations than the ones causing changed cell morphology (0,2 mU – 0,4 mU). The maximum IL-8 secreted was also lower for the purified king crab trypsin compared to purified sardine trypsin (9-fold vs 18-fold) and purified salmon trypsin (20-fold) (Larsen *et al*., 2008).
Heat-inactivation completely abolished the IL-8 response to both trypsins (Fig. 1a, supplementary data). This confirms that the observed IL-8 secretion was dependent on the proteolytic activity of both enzymes.

Purified sardine and king crab trypsin stimulates generation of NF-κB, but in a different pattern compared to subsequent IL-8 secretion

Our previous research has shown that purified salmon trypsin stimulates DNA-binding of activated NF-κB (Larsen *et al*., 2008). Based on this knowledge we wanted to investigate if purified sardine and king crab trypsin behaved similarly. The results shown in figure 1b demonstrate that both trypsins are capable of inducing NF-κB-driven luciferase activity in the A549/NF-κB-luc cells. Maximum response was seen with 0,4 mU for both enzymes with a 9,2-fold increase compared to untreated cells for the purified sardine trypsin and a 13-fold increase for purified king crab trypsin. In contrast to the IL-8 response, the maximum NF-κB generated was higher for the purified king crab trypsin compared to the purified sardine trypsin. We also registered that the higher concentrations of purified king crab trypsin were able to stimulate NF-κB activation without subsequent generation of IL-8.

Secretion of IL-8 and activation of NF-κB in A549/NF-κB-luc cells by purified sardine and king crab trypsins are, at least partly, due to activation of PAR-2

Based on the divergence in the NF-κB and IL-8 dose-response results, we wanted to investigate if the observed IL-8 secretion and NF-κB response was due to activation of PAR-2 with respect to both trypsins as previously shown for salmon trypsin (Larsen *et al*., 2008). We used specific siRNA to knock down the expression of endogenous PAR-2 in A549/NF-κB-luc cells. A mixture of three siRNAs targeting the PAR-2 gene is previously shown to reduce the expression of endogenous PAR-2 transcripts at 24 hours post-transfection in A549 cells with 91% compared to non-transfected cells using real-time PCR (Larsen *et al*., 2008). Stimulation of mock-transfected (receiving negative control) and PAR-2 knockdown cells with PAR-2 agonist peptide, purified sardine trypsin and purified king crab trypsin showed a reduced level of IL-8 secretion in the cells receiving siRNA targeting PAR-2 compared to the negative control (Fig 2). The amount IL-8 secreted was reduced with 52% in the PAR-2 agonist peptide stimulated cells, 0%, 17%, and 34% (0,02 mU, 0,2 mU, and 0,4 mU) in the cells stimulated with purified sardine trypsin, and 29% and 31% (0,02 mU and 0,2 mU) in the cells stimulated with purified king crab trypsin. The highest concentration of purified king crab trypsin (0,4 mU) yielded no IL-8 secretion.
We also observed a reduced level of NF-κB-driven luciferase activity in the cells receiving siRNA targeting PAR-2 compared to the negative control (Fig 3). The amount NF-κB generated was reduced with 57% in the PAR-2 agonist peptide stimulated cells, 0%, 24%, and 38% (0,02 mU, 0,2 mU, and 0,4 mU) in the sardine trypsin stimulated cells, and with 32%, 31%, and 49% (0,02 mU, 0,2 mU, and 0,4 mU) in the cells stimulated with purified king crab trypsin.

IL-8 secretion by purified sardine and king crab trypsin stimulated A549/NF-κB-luc cells is dependent on p38 and MEK activity, as well as NF-κB generation, but probably through separate pathways

Research indicates that maximal IL-8 protein expression requires activation of NF-κB as well as activation of the MAP kinases ERK, JNK, and p38 (Li *et al*., 2002). In order to investigate the contribution of MEK/ERK , p38 and NF-κB to the observed secretion of IL-8 and by this to address the observed lack of IL-8 secretion following the generous NF-κB induction in cells stimulated by higher concentrations of purified king crab trypsin, we examined the effects of U0126 (MEK inhibitor), SB202190 (p38 inhibitor), and BAY 11-7082 (NF-κB inhibitor) in PAR-2 agonist peptide, sardine and king crab stimulated A549/NF-κB-luc cells.
The use of 10 μM U0126 reduced the secretion of IL-8 after stimulation with the PAR-2 agonist peptide SLIGRL-NH_2 (25 μM) with 86%, while the NF-κB level was almost unchanged with a slight decrease of 9% (Fig. 4). Application of U0126 also reduced the secreted IL-8 level after stimulation with purified sardine trypsin with 81% and 86% (0,2 mU and 0,4 mU), while the NF-κB levels showed a slight increase with 7% and 9%. U10126 reduced the secretion of IL-8 following king crab trypsin

stimulation with 65% (0,02 mU) (stimulation with 0,4 mU king crab trypsin yields no detectable IL-8), while the NF-κB levels increased with 25% and 16% (0,02 mU and 0,4 mU).

Application of the p38 inhibitor SB202190 (10 μM) reduced the secreted IL-8 level with 52% from the PAR-2 agonist peptide stimulated cells, while the NF-κB level was reduced with 46% (Fig. 5). SB202190 attenuated the secretion of IL-8 from purified sardine trypsin stimulated cells with 45% and 35% (0,2 mU and 0,4 mU). The levels of NF-κB were reduced with 49%, and 44% respectively. In the king crab stimulated cells the SB202190 reduced the secretion of IL-8 with 40% (0,02 mU) (stimulation with 0,4 mU king crab trypsin yields no detectable IL-8). The levels of NF-κB were reduced with 53% (0,02 mU and 0,4 mU).

The use of a NF-κB inhibitor (BAY 11-7082, 100 μM) reduced the secretion of IL-8 from the PAR-2 peptide stimulated cells with 99%, the purified sardine trypsin stimulated cells with 98% and 99% (0,2 mU and 0,4 mU), and from the purified king crab stimulated cells with 98% (0,02 mU) (stimulation with 0,4 mU king crab trypsin yields no detectable IL-8). BAY 11-7082 completely abolished the generation of NF-κB at a concentration of 100 uM (Fig. 2, supplementary data).

Discussion

Although a precise function for PAR-2 in the airways is currently uncertain, several lines of evidence support a role for PAR-2 in the progression of inflammatory diseases (Vergnolle, 2009). Inhalation of biological particles containing exogenous proteases may produce inflammatory responses in the airways due to activation of PAR-2 mediated reactions. PAR-2 activation on lung epithelial cells is responsible for the release of numerous mediators of inflammation such as IL-8, IL-6, prostaglandin E_2, matrix-metalloprotease-9 (MMP-9), and granulocyte macrophage-colony stimulating factor (GM-CSF) (Vliagoftis *et al*., 2000, 2001; Asokananthan *et al*., 2002). In this study, we have shown that purified sardine and king crab trypsins 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 (Larsen *et al.,* 2008). The secretion of IL-8 occurred in a concentration-dependent manner with regards to both trypsins tested, but the optimal concentration of enzyme producing maximal secretion of IL-8 differed. Purified sardine trypsin was most potent at 0,2 mU while the purified king crab trypsin stimulated maximal IL-8 secretion already at 0,02 mU, yielding no secretion of IL-8 in the 0,2 mU concentration. Additionally, the two trypsins differed with respect to maximal levels of IL-8 induced. The sardine trypsin was most effective generating twice as much IL-8 secreted in the maximal concentration compared to king crab trypsin. This resulted in diverging dose-response curves for the two enzymes. As the enzymes were equally effective when measured by the serine protease assay, the dose-response results were somewhat unexpected.

We have previously shown that knockdown of PAR-2 expression by specific siRNA almost completely abolishes the dose-dependent release of IL-8 caused by stimulation of A549 cells with different concentrations of purified salmon trypsin, demonstrating that salmon trypsin induce expression of IL-8 via activation of PAR-2 (Larsen *et al.,* 2008). By incorporating the selective PAR-2 agonist peptide to the cell stimulation assays we show that activation of PAR-2 make a major contribution to the observed secretion of IL-8. The use of siRNA targeting PAR-2 significantly reduced the secretion of IL-8 from PAR-2 knockdown cells with 52% following stimulation with PAR-2 agonist peptide, SLIGRL-NH_2. The secretion of IL-8 was reduced with 34% at the most in the sardine and king crab trypsin stimulated PAR-2 knockdown cells. Although not significant, the results indicate that both trypsins are capable of activating PAR-2. The increased residual level compared to untreated cells may be due to incompletely silencing of the PAR-2 receptor as 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 previously used (Larsen *et al.,* 2008). There might also be a possibility of the purified sardine/crab trypsins activating other receptors/ signalling pathways leading to increased secretion of IL-8. Recent data from our collaborators imply that both sardine and king crab trypsin activate hPAR-1 as well as rPAR-4 (Hollenberg MD, personal communication). 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 (Asokananthan *et al*., 2002).

In addition to IL-8 secretion, we also wanted to address the question whether the trypsins (sardine/king crab) were able to activate NF-κB through PAR-2 activation, as reported for other serine proteases in general (Kanke *et al.,* 2001; Adam *et al.,* 2006; Kida *et al.,* 2007, 2008) and purified salmon trypsin in particular (Larsen *et al.,* 2008). Both trypsins induced generation of NF-κB in human airway epithelial cells, an effect at least partly mediated by PAR-2 activation since knockdown of the receptor displayed reduced levels of NF-κB generated (0 – 38% in sardine trypsin stimulated cells, and 31 – 49% in king crab trypsin stimulated cells). In PAR-2 agonist peptide stimulated cells the level of NF-κB was significantly reduced with 57% in PAR-2 knockdown cells. Based on these result we are able to conclude that both sardine and king crab trypsin are able to increase the secretion of IL-8 and generate activated NF-κB in human airway epithelial cells, at least partially, through PAR-2 activation.

The receptor knockdown studies confirmed PAR-2 activation but revealed no obvious differences between the sardine and king crab trypsins that may lead to the dissimilar IL-8 dose-response relationships. Some proteases, including the PAR-2 agonist tryptase, 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 (Molino *et al.,* 1997; Ossovskaya and Bunnett, 2004). Regarding tryptase, the cleavage of the inactivation site is shown to be concentration-dependent and occurs at 100 nM compared to the cleavage at the activation site which can be detected already at 1 nM of tryptase. Differences in capacity to cleave the PAR-2 N-terminal tail creating the activating tethered ligand may be a possible cause for variation in the dose-response relationship for the trypsins. Both trypsins may be capable of cleaving PAR-2 at activation and disabling sites, but the enzyme concentration at which these reverse effects occur may vary creating the observed differences in dose-response relationship.

When comparing the results from the reporter gene assays measuring NF-κB with the concentration-dependent release of IL-8, we observed that the concentrations of purified sardine trypsin that yielded the highest induction of NF-κB also lead to the highest release of IL-8. On the contrary, the purified king crab trypsin showed the highest induction of NF-κB-driven luciferase activity in an enzyme concentration (0,4 mU) that yielded no detectable IL-8. For the purified sardine trypsin we found the dose-response curve for IL-8 to follow the generated levels of NF-κB, while for the purified king crab trypsin the NF-κB response in the higher enzyme concentrations (0,2 – 2 mU) did not generate secretion of IL-8 (see Fig. 1).
Earlier researches have shown that increased expression of IL-8 is mediated by transcriptional regulation involving the transcription factors NF-κB, NF-IL6 (C/EBP-β), and AP-1, with NF-κB being essential. Unlike the NF-κB site, the AP-1 and NF-IL-6 sites are not essential for induction but are required for maximal gene expression (Matsusaka *et al.,* 1993; Kunsch *et al.,* 1994; Mukaida *et al.,* 1994; Garofalo *et al.,* 1996; Mastronarde *et al.,* 1996, 1998; Blackwell and Christman 1997; Brasier *et al.,* 1998; Hoffmann *et al.,* 2002). Our previous work has established that purified salmon trypsin promotes DNA-binding of NF-κB via PAR-2 (Larsen *et al.,* 2008). A possible explanation for the diverging results in the king crab stimulation assays could be that purified king crab trypsin activate

PAR-2 in the lower enzyme concentration range, resulting in generation of activated NF-kB and secretion of IL-8, while in the higher enzyme concentrations the king crab trypsin activates an unknown receptor/separate signalling pathways that induces NF-kB activation but does not stimulate IL-8 secretion. The generated NF-kB may very well cause transcription of different target genes besides IL-8, as NF-κB is a transcription factor known to regulate the expression of over 200 immune, growth, and inflammation genes (Aggarwal, 2004) and is activated by a large number of receptors and pathways (Bonizzi and Karin, 2004; Bassères and Baldwin, 2006).This scenario might be partly true. However, the results from our siRNA assays show that the increased levels of NF-κB following stimulation with king crab in the higher enzyme concentrations (0,2 mU and 0,4 mU) also involves PAR-2 activation as we observe a reduction of 31 – 35% in PAR-2 knockdown cells. The fact that this amount of activated NF-κB, although proven to be partly due to PAR-2 activation, did not lead to secretion of IL-8 as observed in the lower enzyme concentrations puzzled us. Theoretically, as previously mentioned, purified king crab trypsin might be able to cleave PAR-2 both at activation and inactivation sites, but an inactivation of the receptor does only explain the lack of IL-8 secretion and not the increased amounts of NF-κB. Besides inactivation, concentration-dependent differences in the capability to cleave the PAR-2 N-terminal end might activate separate PAR-2 coupled signalling pathways with different end result devoid of IL-8 secretion. However, the question why the apparent PAR-2 mediated NF-κB generation translates into increased secretion of IL-8 only in the lower enzyme concentrations of king crab trypsin (0,004 – 0,01 mU) and not in the higher range (0,2 – 2 mU) needs further investigation to be answered.

According to Hoffmann and co-workers (2002) maximal IL-8 amounts are generated by a combination of three different mechanisms: first, de-repression of the gene promoter; second, transcriptional activation of the gene by NF-κB and JUN-N-terminal protein kinase pathways; and third, stabilization of the mRNA by the p38 mitogen-activated pathway. In human airway epithelial cells maximal TNFα-induced IL-8 protein expression is shown to require activation of NF-κB as well as activation of the MAP kinases ERK, JNK, and p38 (Li *et al*., 2002).

In an attempt to reveal possible differences in the PAR-2 activating potential between the trypsins investigated, we have 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. ERK activation has been demonstrated to be required for IL-8 mRNA or protein expression in A549 lung epithelial cells (Chen *et al.,* 2000) and PAR-2 has been shown to activate ERK in the airways by stimulating human bronchial epithelial cells (16HBE14o) with a selective PAR-2 peptide (SLIGKV) (Page *et al.,* 2003). In line with this research we observed a pronounced reduction in the secreted levels of IL-8 when inhibiting MEK/ERK in PAR-2 agonist peptide (SLIGRL-NH_2), purified sardine and king crab trypsin stimulated A549 cells. The NF-κB levels remained almost unchanged in the PAR-2 agonist peptide and sardine trypsin stimulated cells, indicating that the contribution of MEK/ERK to IL-8 secretion is a NF-κB-independent process. In the king crab trypsin stimulated cells the NF-κB level showed an increase of 16 – 25% in the inhibitor treated cells, but these results were not found to be significant. The U0126 inhibitor studies showed that activation of MEK/ERK contribute to the secretion

of IL-8 from human airway epithelial cells following stimulation with sardine and king crab trypsins, but revealed no significant differences between the two trypsins.

Activation of PAR-2 by PAR-2 agonist peptide (SLIGKV) and trypsin is shown to increase p38 mitogen-activated protein kinase activity in a human keratinocyte cell line (NCTC2544) (Kanke *et al.,* 2001). P38 is primarily believed to regulate the IL-8 protein expression at a post-transcriptional level. Li and co-workers (2002) have shown that the use of a chemical p38 inhibitor (SB202190) failed to attenuate transcription from the IL-8 promoter, but significantly reduced TNFα-induced IL-8 protein abundance from human bronchial epithelial cells. As for IL-8, NF-κB also play a crucial role in IL-6 gene expression and former studies have shown that inhibition of the p38 MAPK pathway abrogates TNF-mediated IL-6 gene expression without affecting the levels of TNF-induced NF-κB release and DNA-binding (Beyaert *et al.,* 1996). However, there are studies that indicate involvement of p38 in NF-κB-dependent processes as well. In murine fibrosarcoma cells (L929sA), the TNF-activated p38 pathway contributes to transcriptional activation by modulating the transactivation capacity of the NF-κB p65 subunit (Vanden Berghe *et al.,* 1998). Furthermore, in primary human dendritic cells, p38 mitogen-activated protein kinase-dependent phosphorylation and phosphoacetylation of histone H3 is shown to be induced by inflammatory stimuli. This selectively occurred on the promoters of cytokine and chemokine genes. P38 activity was required to enhance the accessibility of the cryptic NF-κB binding sites contained in H3 phosphorylated promoters, which indicates that p38-dependent H3 phosphorylation may mark promoters for increased NF-κB recruitment (Saccani *et al.,* 2002).

In line with former inhibitor-studies we found that the p38 inhibitor reduced the secreted levels of IL-8 from PAR-2 agonist peptide (52%) and trypsin stimulated (sardine 35 – 45% and king crab 40%) A549 cells, but in contrast to the referred studies we observed a reduced level of NF-κB following p38 inhibition both in peptide and trypsin stimulated cells (PAR-2 agonist peptide 46%, sardine trypsin 44 – 49%, and king crab trypsin 51 – 56%). Although our SB202190 inhibitor studies indicate that p38 contribute to the regulation of secreted IL-8 from human airway epithelial cells as well as activation of NF-κB following stimulation with sardine and king crab trypsins, no significant differences were observed between the two trypsins. The role of NF-κB being essential for IL-8 production were confirmed as the use of a NF-κB-inhibitor completely abolished the secretion of IL-8 from A549 cells following stimulation with PAR-2 agonist peptide and both trypsins.

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 in addition to a probable regulation of the IL-8 protein expression at a post-transcriptional level as reported by others, may influence upon activation, nuclear translocation and/or DNA binding of activated NF-κB.

Besides the slight increase in NF-κB activity in MEK-inhibitor treated king crab trypsin stimulated A549 cells, the 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.

Research by Ramachandran and co-workers (2009) has shown that PAR-2 exhibit functional selectivity and that the proteolytically revealed tethered ligand (TL) sequence(s) and the mode of its presentation to the receptor (tethered vs. soluble) can confer biased signalling. Besides influencing upon the activation of different signalling pathways, substitution of amino acids in the activating sequence may modulate the strength of a given signal. In addition to the Ser^{37}-Leu^{38} in the tethered ligand, the Arg^{5} (corresponding to Arg^{41} in the TL) in the PAR-2 agonist peptide has been shown to be important for the peptide's potency (Al-Ani *et al.,* 2002, 2004). Changing this positive Arg^{5} in SLIG**R**L to a neutral alanine or a negative glutamatic acid creating SLIG**A**L or SLIG**E**L markedly reduces the peptides potency to cause intracellular Ca^{2+} signalling. This residue (Arg^{5}) is from receptor chimera studies in PAR-1 suggested to interact with the negative Glu^{260} in the second extracellular loop of PAR-1 during receptor activation (Gerszten *et al.,* 1994; Nanevicz *et al.,* 1995). Since these amino acids are conserved in PAR-2 an Arg^{5}-Glu^{260} interaction might operate in recognition of the PAR-2 agonist peptide SLIGRL by the receptor. The study conducted by Al-Ani and co-workers (2002) indicate that changes in electrostatic potential seems to be an important aspect in the binding of agonist to PAR_2. Whether these changes in electrostatic potential only results in modulation of the strength of a given intracellular signal or whether they may contribute to the activation of different signalling pathways resulting in functional selectivity following PAR-2 activation is presently unknown. Molecular modelling conducted by our group shows that the surface of king crab trypsin displays a more negative electrostatic potential compared to salmon and bovine trypsins (Larsen *et al.* – manuscript). The modelling studies also suggest at least 3 divergent positions located near the substrate binding pocket that may affect binding of substrate to PAR-2. 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} 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. It might be a possibility that the differences observed in net charge and the molecular size/structure in king crab trypsin compared to other fish trypsins may cause a concentration-dependent biased signalling giving rise to the observed differences in secretion of IL-8 and generation of activated NF-kB, but this issue needs to be further explored.

In summary, we have demonstrated that purified sardine and king crab trypsins induce secretion of IL-8 from human airway epithelial cells, at least partially, via activation of PAR-2. We can also conclude that sardine and king crab trypsin promotes DNA-binding of NF-κB, at least partially, through PAR-2 activation, and that generation of NF-κB is essential for secretion of IL-8 protein following trypsin stimulation. The MAP kinases MEK/ERK and p38 are both activated by sardine and king crab trypsins and contribute to the increased secretion of IL-8 by NF-κB independent (MEK/ERK) and seemingly NF-κB-dependent (p38) processes.

From this work we are not able to make conclusions regarding the differences in IL-8 and NF-κB dose-response relationships, but the results 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 will be important in understanding PAR-2 mediated signalling events in human airway epithelial cells.

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Figures and legends

Fig. 1

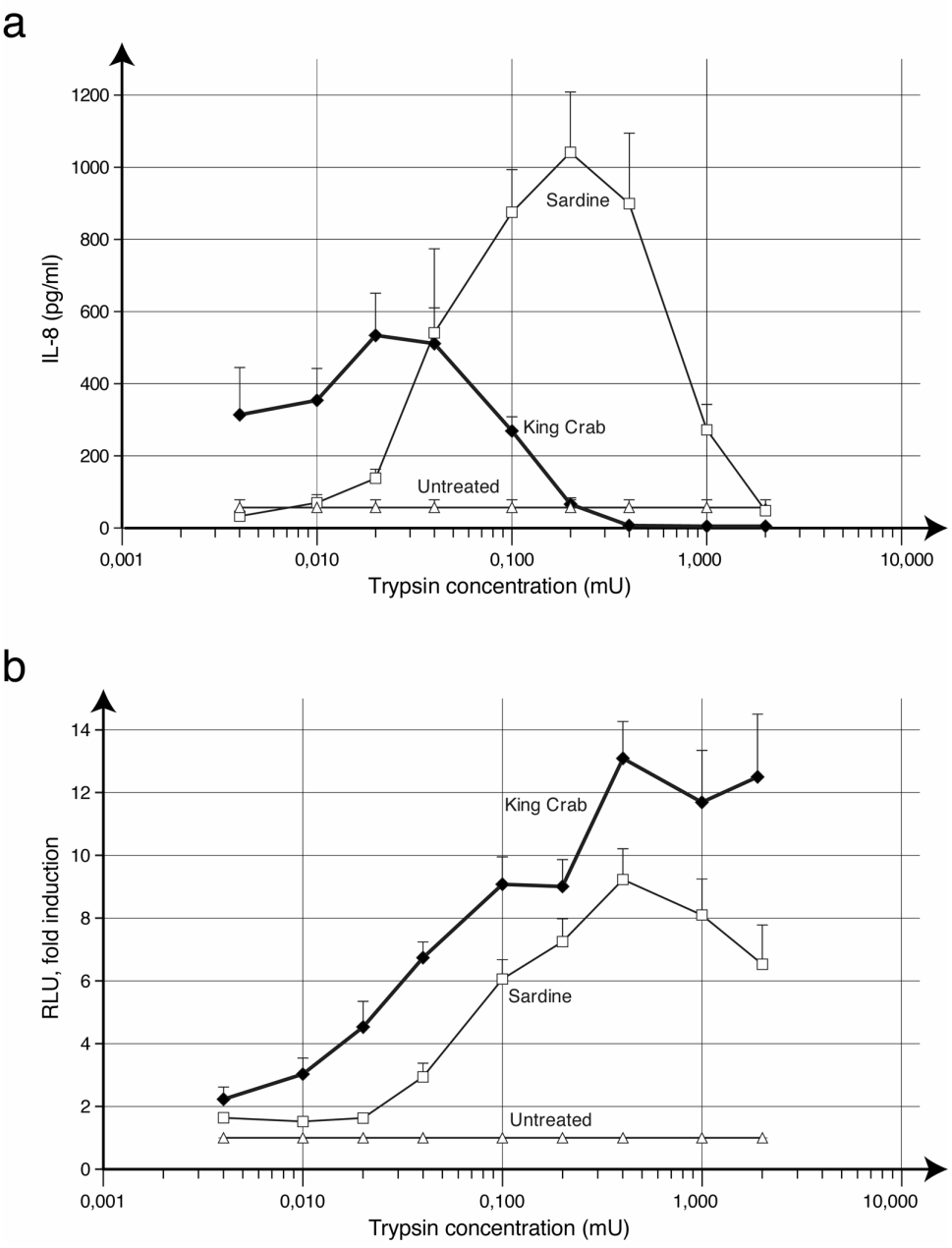

Figure 1

Dose-response relationships of IL-8 and NF-κB in human airway epithelial cells following sardine and king crab trypsin stimulation. A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes, grown to 80-90% confluency and kept in serum-free medium for 24 hours prior to incubation with the indicated concentrations of purified sardine and king crab trypsin. (A) The

supernatant were harvested 6 h later and analyzed for the presence of IL-8 by ELISA. (B) For the analysis of NF-κB-driven luciferase-expression the cells were lysed and harvested after removal of the supernatant. Lysates were analysed for luciferase activity and normalized against the total protein concentration. The results are given as pg/ml supernatant (a) or fold induction above basal levels in untreated cells (b). Data are expressed as mean ± S.E., n = 6 – 10 from at least three separate experiments in (a) and n = 4 – 8 from at least two separate experiments in (b). RLU (relative light units).

Fig. 2

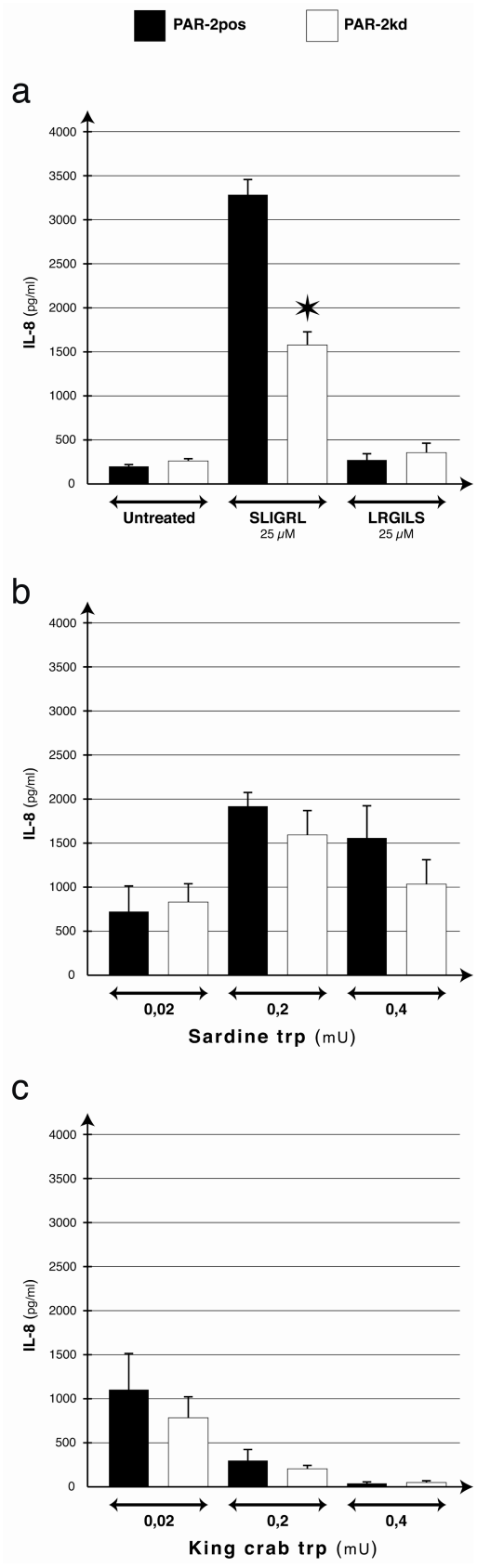
PAR-2pos
PAR-2kd
a
IL-8 (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
Untreated
SLIGRL
25 μM
LRGILS
25 μM
b
IL-8 (pg/ml)
0,02
0,2
0,4
Sardine trp (mU)
c
IL-8 (pg/ml)
0,02
0,2
0,4
King crab trp (mU)

Figure 2. **Inhibition of IL-8 secretion from A549/NF-κB-luc cells using siRNA against PAR-2.** A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes. Forty-eight hours later the cells were transfected with a mixture of three different siRNAs (total of 40 nM) targeting hPAR-2. Cells not receiving siRNA targeting PAR-2 were transfected with a negative control siRNA. The cells were kept in serum-free standard medium for 24 h until stimulation with PAR-2 agonist peptide (SLIGRL-NH_2), inactive control peptide (LRGILS-NH_2) (a), or the indicated concentrations of sardine (b) and king crab trypsins (c). The supernatant were harvested 6 h later and analyzed for the presence of IL-8 by ELISA. Data are expressed as mean ± S.E., n = 4 – 6 from at least 2 separate experiments. (* different from PAR-2 positive cells, $p < 0{,}02$).

Fig. 3

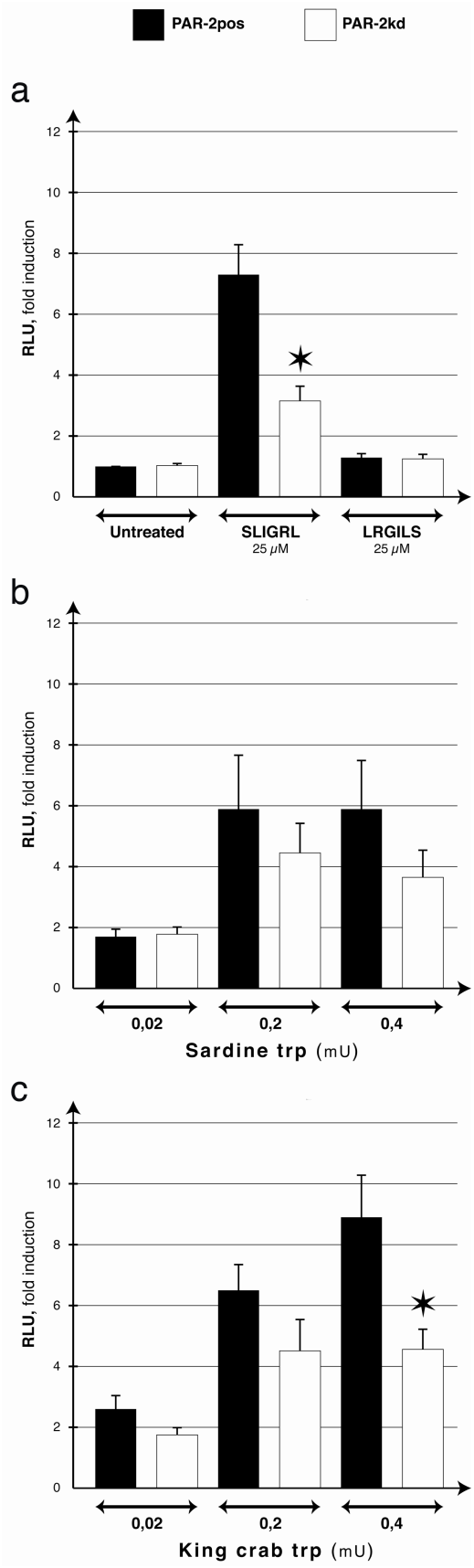
PAR-2pos
PAR-2kd
a
RLU, fold induction
0
2
4
6
8
10
12
Untreated
SLIGRL
25 μM
LRGILS
25 μM
b
RLU, fold induction
0,02
0,2
0,4
Sardine trp (mU)
c
RLU, fold induction
0,02
0,2
0,4
King crab trp (mU)

Figure 3. **Inhibition of NF-kB activation in A549/NF-κB-luc cells using siRNA against PAR-2.** A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes. Forty-eight hours later the cells were transfected with a mixture of three different siRNAs (total of 40 nM) targeting hPAR-2. Cells not receiving siRNA targeting PAR-2 were transfected with a negative control siRNA. The cells were kept in serum-free standard medium for 24 h until stimulation with PAR-2 agonist peptide (SLIGRL-NH_2), inactive control peptide (LRGILS-NH_2) (a), or the indicated concentrations of sardine (b) and king crab trypsin (c). After removal of the supernatant the cells were lysed and harvested. Lysates were analysed for luciferase activity and normalized against the total protein concentration. The results are given as fold induction above basal levels in untreated cells. Data are expressed as mean ± S.E., n = 4 – 6 from at least 2 separate experiments. RLU (relative light units), * (different from PAR-2 positive cells, $p < 0,02$).

Fig. 4

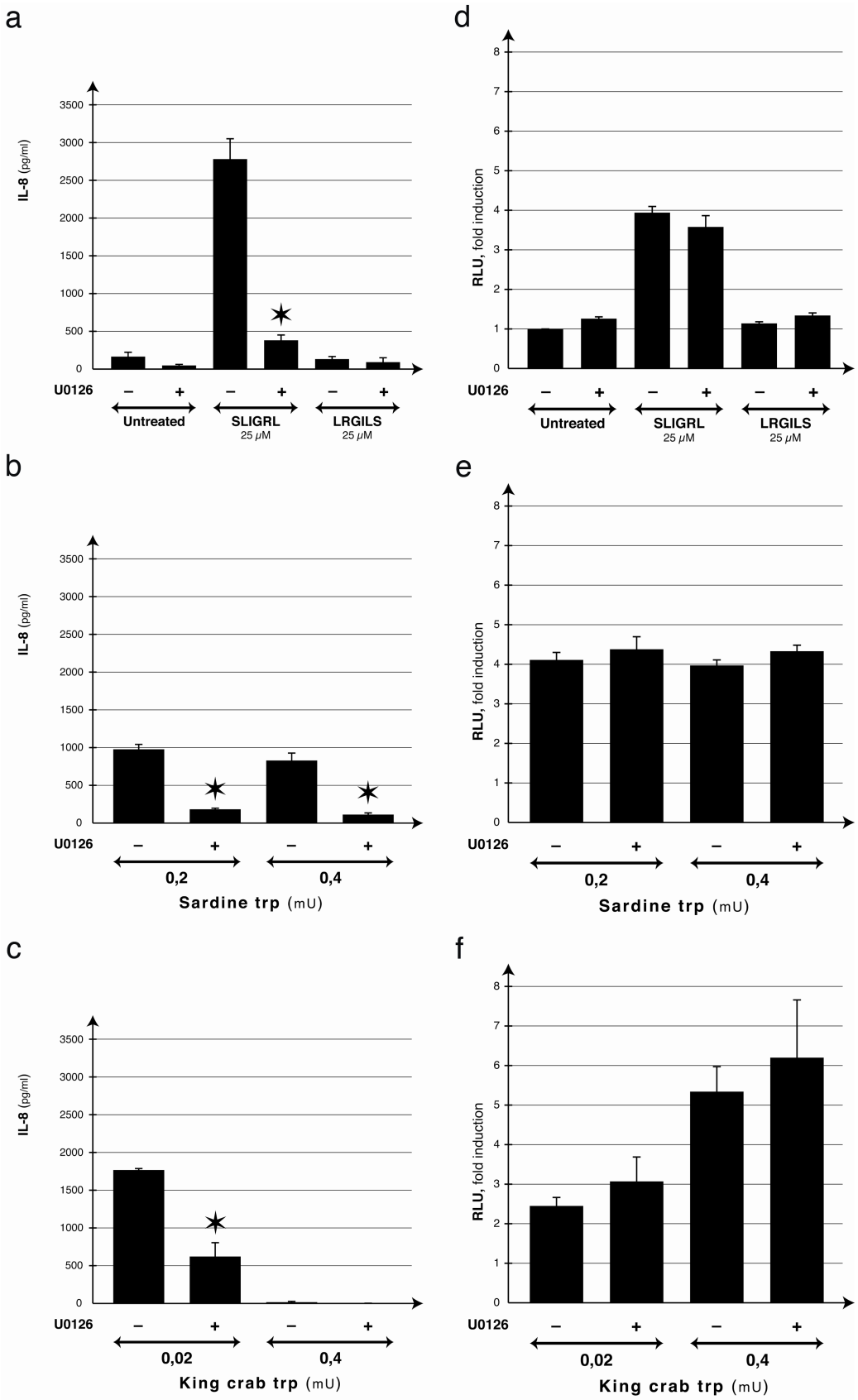
a
IL-8 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
U0126
– + – + – +
Untreated
SLIGRL 25 μM
LRGILS 25 μM
d
RLU, fold induction
8
7
6
5
4
3
2
1
0
U0126
– + – + – +
Untreated
SLIGRL 25 μM
LRGILS 25 μM
b
IL-8 (pg/ml)
U0126
– + – +
0,2
0,4
Sardine trp (mU)
e
RLU, fold induction
U0126
– + – +
0,2
0,4
Sardine trp (mU)
c
IL-8 (pg/ml)
U0126
– + – +
0,02
0,4
King crab trp (mU)
f
RLU, fold induction
U0126
– + – +
0,02
0,4
King crab trp (mU)

Figure 4. **Inhibition of MEK activity in human airway epithelial cells reduces secretion of IL-8 but not generation of NF-κB.** A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes, grown to 80-90% confluency and kept in serum-free medium for 24 hours. Inhibitor (10 μM U0126 in DMSO) or DMSO (as negative control in cells not receiving inhibitor) was added to the wells 1 hour prior to stimulation with PAR-2 agonist peptide (SLIGRL-NH_2, 25 uM), inactive control peptide (LRGILS-NH_2, 25 uM), sardine and king crab trypsins. (a – c) The supernatant were harvested 6 h later and analyzed for the presence of IL-8 by ELISA. (d – f) For the analysis of NF-κB-driven luciferase-expression the cells were lysed and harvested after removal of the supernatant. Lysates were analysed for luciferase activity and normalized against the total protein concentration. The results are given as pg/ml supernatant (a – c) or fold induction above basal levels in untreated cells (d – f). Data are expressed as mean ± S.E., n = 12 from six separate experiments in (a), n = 4 – 8 from at least two separate experiments in (b – c), n = 10 from five separate experiments in (d) and n = 4 – 6 from at least two separate experiments in (e – f). RLU (relative light units), * (different from non-inhibitor treated cells, $p < 0,02$).

Fig. 5

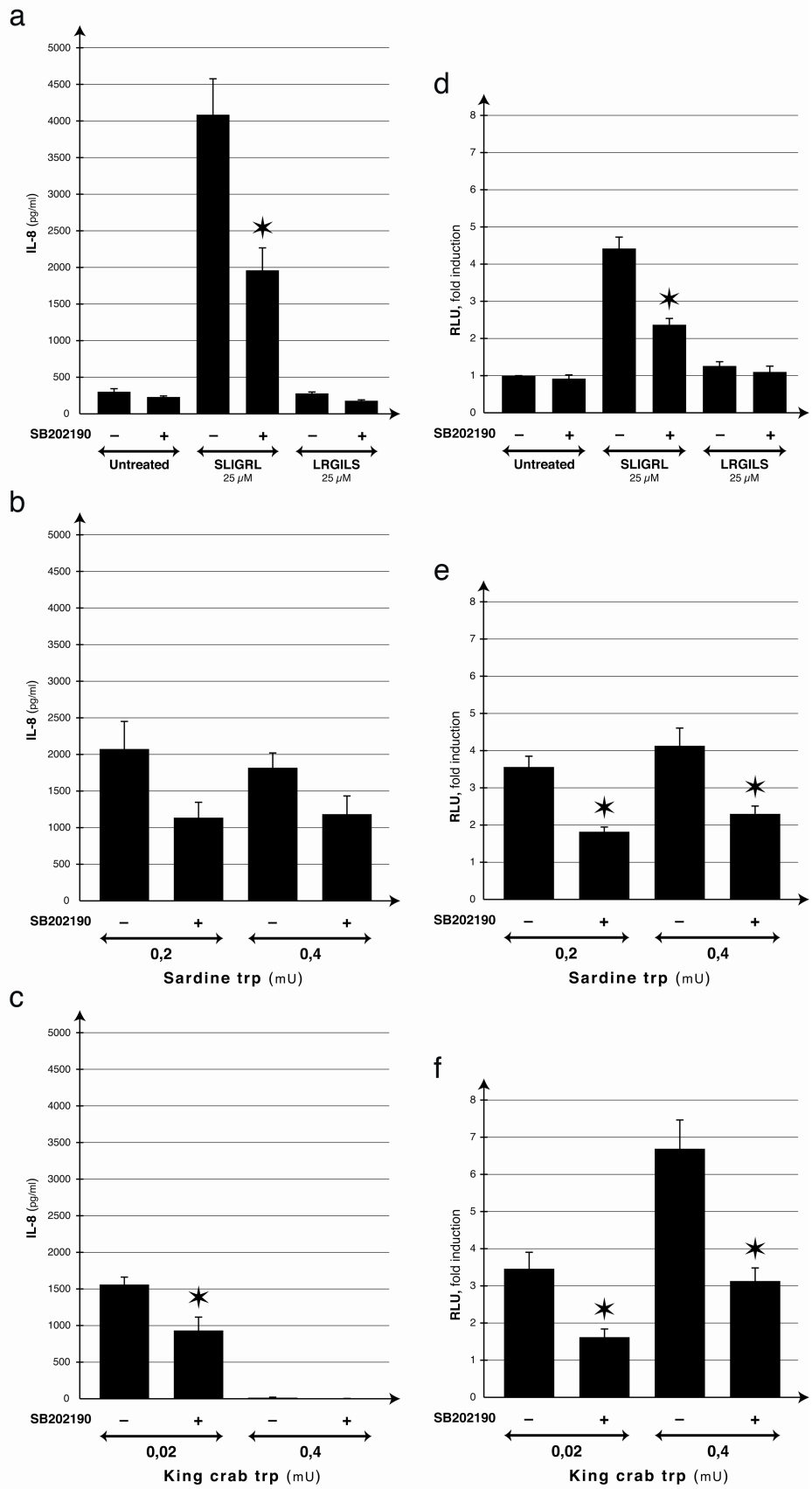
a
IL-8 (pg/ml)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
SB202190 – + – + – +
Untreated
SLIGRL 25 μM
LRGILS 25 μM
d
RLU, fold induction
8
7
6
5
4
3
2
1
0
SB202190 – + – + – +
Untreated
SLIGRL 25 μM
LRGILS 25 μM
b
IL-8 (pg/ml)
SB202190 – + – +
0,2
0,4
Sardine trp (mU)
e
RLU, fold induction
SB202190 – + – +
0,2
0,4
Sardine trp (mU)
c
IL-8 (pg/ml)
SB202190 – + – +
0,02
0,4
King crab trp (mU)
f
RLU, fold induction
SB202190 – + – +
0,02
0,4
King crab trp (mU)

Figure 5. **Inhibition of p38 activity in human airway epithelial cells reduces secretion of IL-8 and generation of NF-κB.** A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes, grown to 80-90% confluency and kept in serum-free medium for 24 hours. Inhibitor (10 μM SB202190 in DMSO) or DMSO (as negative control in cells not receiving inhibitor) was added to the wells 1 hour prior to stimulation with PAR-2 agonist peptide (SLIGRL-NH_2, 25 uM), inactive control peptide (LRGILS-NH_2, 25 uM), sardine or king crab trypsins. (a – c) The supernatant were harvested 6 h later and analyzed for the presence of IL-8 by ELISA. (d – f) For the analysis of NF-κB-driven luciferase-expression the cells were lysed and harvested after removal of the supernatant. Lysates were analysed for luciferase activity and normalized against the total protein concentration. The results are given as pg/ml supernatant (a – c) or fold induction above basal levels in untreated cells (d – f). Data are expressed as mean ± S.E., n = 6 from three separate experiments in (a and d), n = 4 – 6 from at least two separate experiments in (b, c, e, and f). RLU (relative light units), * (different from non-inhibitor treated cells, $p < 0,02$).

Supplementary data

Fig. 1

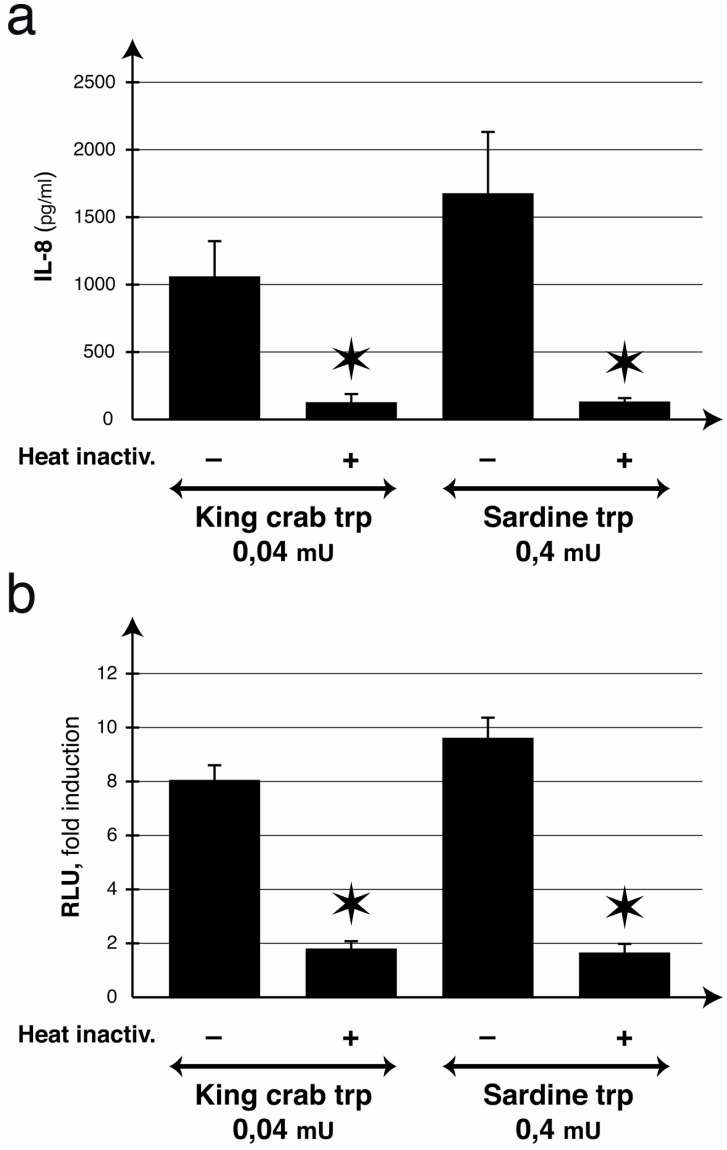

Figure 1. **Secretion of IL-8 and generation of NF-κB by king crab and sardine trypsins in human airway epithelial cells are dependent on protease activity.** A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes, grown to 80-90% confluency and kept in serum-free medium for 24 hours prior to incubation with the indicated concentrations of purified sardine and king crab trypsins, intact or heat inactivated by incubation at 95°C for 15 min. (A) The supernatant were harvested 6 h later and analyzed for the presence of IL-8 by ELISA. (B) For the analysis of NF-κB-driven luciferase-expression the cells were lysed and harvested after removal of the supernatant. Lysates were analysed for luciferase activity and normalized against the total protein concentration. The results are

given as pg/ml supernatant (a) or fold induction above basal levels in untreated cells (b). Data are expressed as mean ± S.E., n = 4 from two separate experiments. RLU (relative light units), * (different from active trypsins, $p < 0,02$).

Fig. 2

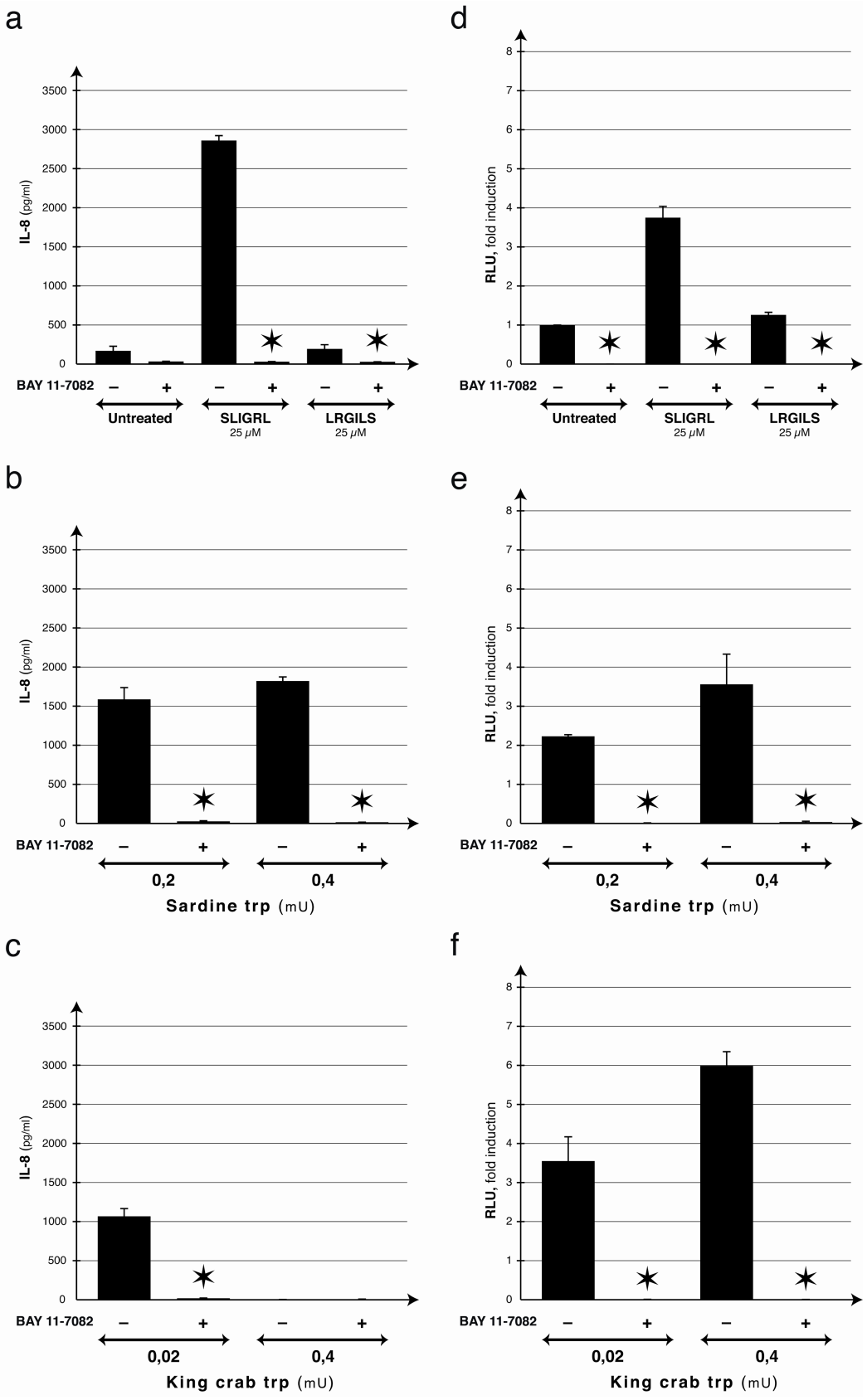
a
IL-8 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
BAY 11-7082 – + – + – +
Untreated
SLIGRL 25 μM
LRGILS 25 μM
d
RLU, fold induction
8
7
6
5
4
3
2
1
0
BAY 11-7082 – + – + – +
Untreated
SLIGRL 25 μM
LRGILS 25 μM
b
IL-8 (pg/ml)
BAY 11-7082 – + – +
0,2
0,4
Sardine trp (mU)
e
RLU, fold induction
BAY 11-7082 – + – +
0,2
0,4
Sardine trp (mU)
c
IL-8 (pg/ml)
BAY 11-7082 – + – +
0,02
0,4
King crab trp (mU)
f
RLU, fold induction
BAY 11-7082 – + – +
0,02
0,4
King crab trp (mU)

Figure 2. **Inhibition of NF-kB abolishes the secretion of IL-8.** A549/NF-κB-luc cells (2 x 10^5 cells/well) were seeded out in 6-well dishes, grown to 80-90% confluency and kept in serum-free medium for 24 hours. Inhibitor (100 µM BAY 11-7082 in DMSO) or DMSO (as negative control in cells not receiving inhibitor) was added to the wells 1 hour prior to PAR-2 agonist peptide (SLIGRL-NH_2), inactive control peptide (LRGILS-NH_2), sardine or king crab trypsins. (a – c) The supernatant were harvested 6 h later and analyzed for the presence of IL-8 by ELISA. (d – f) For the analysis of NF-κB-driven luciferase-expression the cells were lysed and harvested after removal of the supernatant. Lysates were analysed for luciferase activity and normalized against the total protein concentration. The results are given as pg/ml supernatant (a – c) or fold induction above basal levels in untreated cells (d – f). Data are expressed as mean ± S.E., n = 4 from two separate experiments. RLU (relative light units), * (different from non-inhibitor treated cells, $p < 0,02$).