Mixed exposure to bacterial lipopolysaccharide (LPS) and seafood proteases augment inflammatory signalling in an airway epithelial cell model (A549).

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Abstract

Seafood industry workers exhibit an increased prevalence of respiratory symptoms due to exposure to bioaerosols containing a mixture of bioactive agents. In this study a human pulmonary epithelial cell model (A549) was exposed to mixtures of bacterial lipopolysaccharide (LPS) and either protease-activated receptor-2 (PAR-2) agonists SLIGKV-NH_2, purified salmon (*Salmo salar)* trypsin or purified king crab (*Paralithodes camtschaticus*) trypsin. The inflammatory response was measured based on nuclear factor kappa B (NF-κB) activation of transcription in a luciferase reporter gene assay and interleukin-8 (IL-8) secretion in an enzyme-linked immunosorbent assay (ELISA). We observed that mixtures of SLIGKV-NH_2 or trypsins with LPS augmented the activation of NF-κB and secretion of IL-8. The effect on IL- 8 secretion was synergistic when both trypsins and LPS were used in the lower concentration range. The results demonstrate that exposure to mixtures of agents that are relevant to seafood industry workplaces may lead to increased inflammatory signalling compared to exposure to the individual agents alone. Furthermore, the results indicate that synergism may occur with the combined exposure to seafood trypsins with and LPS, and is most likely to occur when exposure to either agent is low.

1. Introduction

Respiratory symptoms associated with occupational seafood exposure are common, and several studies have reported that workers in the seafood industry are at risk of developing respiratory disorders (Jeebhay and Cartier, 2010; Jeebhay et al., 2001). Workers in this industry are exposed to bioaerosols generated during seafood handling and processing (Jeebhay and Cartier, 2010). The inhalation of bioaerosols containing allergens, enzymes, microorganisms, endotoxins and other bioactive agents is the main cause of the observed respiratory problems (Bang et al., 2005; Shiryaeva et al., 2014).

In occupational settings, workers are exposed to several bioactive substances that co- exist in the bioaerosol mixtures. It is well known that exposure to mixtures may result in combined effects due to interactions between the different substances (Kartono and Maibach, 2006; Wade et al., 2002; Boyd et al., 1990).

Endotoxin is the major constituent of the outer membrane of gram-negative bacteria. The terms endotoxin and lipopolysaccharide (LPS) are sometimes used interchangeably in the literature. However, the term LPS denotes the chemically pure substance that is free from other chemical compounds, whereas, the term endotoxin refers to the toxin as the compound is present the bacterial cell wall. LPS is soluble in water and consists of a lipid A (phosphoglycolipid) component and a polysaccharide component, which are responsible for the toxicity and immunogenicity, respectively (Liebers et al., 2006). Gram-negative bacteria are ubiquitous in nature, and endotoxins are thus abundantly present in the environment. Human environmental exposure to endotoxin is mainly via inhalation. Studies have reported the presence of airborne endotoxin in various occupational environments, such as agricultural work, food industries, the textile industry, saw mills, waste handling and processing, breweries and paper mills (Rylander, 2002; Michel, 2003; al-Dagal and Fung, 1990). In the seafood industry, Gram-negative bacteria are likely to be present in several processes, and endotoxins have been measured in these work environments (Bang et al., 2005; Shiryaeva et al., 2014). Accumulating evidence suggests that exposure to environmental endotoxins is linked to airway inflammation, bronco constriction, decreased lung function, hypersensitive pneumonitis, chronic bronchitis and asthma (Michel, 2003; Michel, 2001; Schwartz et al., 1995; Thorn, 2001; Hernandez et al., 2011). It has been documented that endotoxin induces

the activation, translocation and DNA binding of nuclear factor-kappa B (NF-κB) (Zhang and Ghosh, 2000; Hernandez et al., 2011; Aul et al., 2012; Guha and Mackman, 2001), a key regulator of immune and inflammatory responses via toll like receptors, and trigger the generation of a variety of inflammatory cytokines, TNF-α, growth factors, and matrix metalloproteinases (MMPs) (O'Grady et al., 2001; Michel et al., 2007).

The role of proteases in promoting the synthesis and release of inflammatory mediators such as cytokines, prostanoids, growth factors and MMP has been previously demonstrated (Bang et al., 2009; Larsen et al., 2008; Bhagwat et al., 2014; Lee et al., 2010; Page et al., 2006; Asokananthan et al., 2002; Vliagoftis et al., 2001). Our previous studies have shown that the trypsin present in seafood is capable of eliciting an inflammatory response via the activation of NF-κB and the secretion of interleukin-8 (IL-8) in a human alveolar cell line and of IL-8 and MMP in a skin keratinocytic cell line via a mechanism that involves protease-activated receptor-2 (PAR-2) (Bhagwat et al., 2014; Larsen et al., 2008; Larsen et al., 2011). Studies performed by Rallabhandi et al. (2008) demonstrated that PAR-2 and toll-like receptor-4 (TLR-4) or cooperatively resulted in an enhanced NF-κB response, which suggests an important role for cooperativity in the inflammatory response to mixtures that contain LPS and PAR-2 agonists (Rallabhandi et al., 2008). Other studies have also demonstrated that concurrent exposure to LPS and PAR-2 agonists synergistically augments the inflammatory signals (Ostrowska et al., 2007).

Therefore, in this study, we chose to investigate whether mixtures of LPS with either salmon (*Salmo salar)* trypsin or king crab (*Paralithodes camtschaticus*) trypsin were able to stimulate a cellular response related to inflammation in the human airway epithelial cell line A549 and to determine whether such mixtures had combined effects on the generation of NF-κB and the release of IL-8.

2. Materials and methods

2.1 Materials

A549 cells (ATCC; no CCL-185) were obtained from American Type Culture Collection and the A549-NF-κB-luc cells (RC0002) were obtained from Panomics, CA, USA. Dulbecco's minimum essential medium/Ham's F12 medium (1:1), foetal calf serum, L-glutamine and penicillin-streptomycin were purchased from Gibco, NY, USA. The 12 well plates (4 cm^2) were obtained from Nunc, Thermo Scientific, MA, USA. The PAR-2 agonist peptide SLIGKV-NH_2 (H-Ser-Leu-Ile-Gly-Lys-Val-NH_2) was purchased from Bachem, Bubendorf, Switzerland. The enzyme-linked immunosorbent assay (ELISA) kit was obtained from BD Biosciences, NJ, USA. The purified salmon trypsin was kindly provided by Dr. Nils Peder Willassen (University of Tromsø, Norway) (Outzen et al., 1996), and the king crab trypsin was provided by Dr. Galina N. Rudenskaia (Moscow State University, Russia) (Rudenskaia et al., 1998). LPS from *Escherichia coli* O111:B4, hygromycin B and the non-enzymatic cell dissociation solution were purchased from Sigma-Aldrich, MO, USA. The Dual-light Luciferase Reporter Gene Assay System was obtained from Applied Biosystems, CA, USA, and the Dc Protein Assay kit was purchased from Bio-Rad, CA, USA.

2.2 Cell Culture

The A549 cells, a human pulmonary epithelial cell line, and A549/NF-κB-luc cells, a human pulmonary epithelial cell line that is stably transfected with an NF-κB-binding luciferase reporter construct, were cultured in Dulbecco's minimum essential medium/Ham's F12 medium (1:1) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. In addition, the culture medium for the A549/NF-κB-luc cells was supplemented with 100 µg/ml hygromycin B. The cells were passaged using a non-enzymatic cell dissociation solution.

2.3 Cell Stimulation experiment

A549 cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well. After reaching 80 % confluence, the cells were starved of serum overnight. The cells were then subjected to stimulation with purified salmon trypsin, purified king crab trypsin, or the PAR-2 agonist

peptide SLIGKV-NH_2 and LPS from *E.coli* O111:B4 for 6 hours. The culture supernatant was collected and stored at – 20 ^{o}C.

2.4 Protease activity

The enzymatic activity of the purified salmon and king crab trypsins was analysed with a serine protease assay as previously described (Outzen et al., 1996; Erlanger et al., 1961). Briefly, the kinetic measurement was determined by the hydrolysis of a chromogenic substrate, Na-benzoyl-$_{D\text{-}L}$-arginine 4-nitroanilide hydrochloride (DL-BAPNA). The absorbance was measured spectrophotometrically at 405 nm for 10 minutes at room temperature and expressed as Units/ml (U/ml). One activity unit was defined as 1 µmol of substrate hydrolysed per minute using an extinction coefficient of 8800 M^{-1} cm^{-1}.

2.5 Quantitative analysis of IL-8 secretion

The amount of IL-8 in the cell culture supernatant was measured with an ELISA kit according to the protocol described in the manufacturer's instructions. The absorbance was measured with an iEMS Multiscan EX (Thermo Labsystems). The amount of IL-8 produced was calculated and expressed as pg/ml.

2.6 Reporter gene assay

A549/NF-κB-luc cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well. After reaching 80% confluence, the cells were starved of serum overnight. The cells were then subjected to stimulation with purified salmon trypsin, purified king crab trypsin, or the PAR-2 peptide agonist SLIGKV-NH_2 and LPS from *E.coli* O111: B4 for 6 hours. The cells were then lysed, and the luciferase activity in the cell culture lysates was measured with a Dual-Light Luciferase Reporter Gene Assay System according to the manufacturer's instructions. The luciferase activity was measured with a Luminoskan RT dual injection luminometer (Labsystems). The total protein concentration in each cell lysate was measured with a DC Protein Assay and used to normalise the luciferase values.

2.7 Statistical Analysis

The response data were regarded as synergistic when exposure to two combined stimulating agents resulted in response levels that exceeded the sum of the response to the respective agents alone at the same concentrations. Student's ***t*** tests for independent samples (SPSS) were performed to compare the group data. Differences were regarded as significant for p values < 0.05.

3. Results

3.1 LPS enhances the secretion of IL-8 in human pulmonary epithelial cells.

To verify whether LPS (endotoxin) induces the secretion of IL-8 in a human pulmonary epithelial cell model, A549 cells were stimulated with different concentrations of bacterial endotoxin (LPS) for 6 hours. LPS induced the secretion of IL-8 in A549 cells in a dose dependent manner (Figure 1). The maximum stimulation was observed when the A549 cells were stimulated with 100 µg/ml LPS, and the lowest stimulation was observed when the A549 cells were stimulated with 10 µg/ml LPS. Concentrations that were lower than 10 µg/ml LPS did not induce an increase in the levels of IL-8 compared to the basal levels.

3.2 Effect of LPS (endotoxin) and SLIGKV-NH_2 on the secretion of IL-8 in human pulmonary epithelial cells.

To determine whether a combination of LPS (endotoxin) and SLIGKV-NH_2 (a synthetic PAR-2 agonist peptide) had an effect on the level of IL-8 in a human pulmonary epithelial cell line, A549 cells were stimulated with different concentrations of SLIGKV-NH_2 alone or in combination with 10 µg/ml LPS. Figure 2 shows that both SLIGKV-NH_2 and LPS individually lead to an increase in the secretion of IL-8, whereas a mixture of SLIGKV-NH_2 and LPS resulted in a further increase in the levels of IL-8 compared to either of the agents alone.

3.3 Seafood trypsin and LPS synergistically induce the secretion of IL-8 in human pulmonary epithelial cells.

To determine whether LPS (endotoxin) along with purified salmon trypsin or king crab trypsin had a synergistic effect on the induction of IL-8 secretion in a human pulmonary epithelial cell line, A549 cells were stimulated with different concentrations of purified salmon trypsin (0.2, 0.6 mU/ml) or king crab trypsin (0.01, 0.015, 0.02 mU/ml) alone and with mixtures of the same concentrations of salmon or king crab trypsin and LPS (10

µg/ml). The results displayed in Figure 3A and 3B demonstrate that the mixtures of LPS and salmon/king crab trypsin induced an increase in the levels of IL-8. A synergistic effect was observed when the A549 cells were stimulated with a mixture of 10 µg/ml LPS with 0.2 mU/ml salmon trypsin or with 0.01 mU/ml or 0.015 mU/ml king crab trypsin. The maximum synergistic response was observed with the mixtures of 0.2 mU/ml salmon trypsin + LPS and 0.015 mU/ml king crab trypsin + LPS with a 4-fold increase in the IL-8 levels compared to the cells treated with either type of trypsin or LPS alone. Higher concentrations of salmon trypsin in the mixture, 0.6 mU/ml + LPS resulted in a 1.4-fold increase in the IL-8 levels compared to the cells treated with 0.6 mU/ml salmon trypsin alone.

3.4 Effect of variable LPS (endotoxin) concentrations

To determine the role of variable LPS (endotoxin) concentrations in combination with the seafood trypsins, A549 cells were exposed to a mixture of purified salmon trypsin (0.2 mU/ml) or king crab trypsin (0.015 mU/ml) and/or higher concentrations of LPS (25 µg/ml and 50 µg/ml). Figure 4 illustrates that a mixture of different concentrations of LPS and the salmon or king crab trypsins enhanced the levels of IL-8. A synergistic effect was observed when the A549 cells were stimulated with a mixture of 0.015 mU/ml king crab trypsin + 25 µg/ml LPS. When the A549 cells were stimulated with a mixture of 0.2 mU/ml salmon trypsin + 50 µg/ml LPS, we observed an increase in the level of IL-8 compared to the cells treated with LPS and salmon trypsin alone, but this increase was less than the sum of the effects of both agents.

3.5 Mixtures of LPS (endotoxin) with SLIGKV-NH_2, salmon or king crab trypsin stimulate the generation of NF-κB in human pulmonary epithelial cells

To investigate whether the activation of NF-κB is stimulated by the exposure of human pulmonary epithelial cells to a mixture of LPS (endotoxin) together with salmon trypsin

or king crab trypsin, A549/NF-κB-luc cells were exposed to different concentrations of SLIGKV-NH_2 (10 μM), purified salmon trypsin (0.2, 0.6 mU/ml) and king crab trypsin (0.01, 0.015, 0.02 mU/ml) alone and mixtures of the same concentrations of SLIGKV-NH_2 and the purified salmon or king crab trypsins with LPS (10 μg/ml). The results in Figure 5 indicate that the mixture of endotoxin (LPS) with SLIGKV-NH_2, purified salmon trypsin or king crab trypsin stimulate the generation of NF-κB, as did each of the agents alone. The maximum response was generated with a mixture 0.015 mU/ml king crab trypsin + 10 μg/ml LPS with a two-fold increase in NF-κB-driven luciferase activity compared to the effect of purified king crab trypsin alone. Higher concentrations of purified king crab trypsin resulted in an increase in the NF-κB-driven luciferase activity compared to the untreated cells but did not exhibit a significant increase in the generation of NF-κB when used in a mixture of trypsin and LPS. A mixture of purified salmon trypsin (0.6 mU/ml) + 10 μg/ml LPS stimulated the generation of NF-κB with a 1.5-fold increase compared to the cells treated with salmon trypsin alone.

4. Discussion

In this study, we focused on the inflammatory responses of airway epithelial cells elicited by exposure to relevant agents in bioaerosols present in seafood industry work environments. The effects of the agents alone and in combination are highlighted. Seafood proteases and endotoxin (LPS) are anticipated to be relevant exposure mixtures in occupational environments in the seafood industry.

In our previous studies, we demonstrated that seafood proteases are capable of stimulating inflammatory responses in airway epithelial cells via PAR-2. PAR-2 agonists together with LPS have previously been shown to potentiate the stimulation of the inflammatory mediator IL-8 in cell models (Ostrowska et al., 2007).

It has been previously documented that endotoxin (LPS) induces the up-regulation of the secretion of pro-inflammatory mediators such as IL-6, IL-8, TNF- α, and IL-1β (Reddi et al., 2003; Guha and Mackman, 2001; Rylander, 2002; Thorn, 2001). Figure one shows that bacterial LPS also elicits a dose-dependent increase in IL-8 secretion in these A549 cells. Similarly, the ability of proteases to stimulate the secretion of innate inflammatory mediators is well known (Bang et al., 2009; Bhagwat et al., 2014; Larsen et al., 2008; Larsen et al., 2011; Lee et al., 2010; Bhat et al., 2003; Kato et al., 2009; Kauffman et al., 2000). The involvement of PAR-2 in serine protease-mediated up-regulation of inflammatory mediators has been previously documented (Sun et al., 2001; Hong et al., 2004; Page et al., 2006; Bhagwat et al., 2014; Larsen et al., 2008; Larsen et al., 2011). Based on our own previous studies, salmon trypsin as well as king crab trypsin are capable of eliciting a dose-dependent increase in NF-κB activation and IL-8 secretion via PAR-2 in A549 cells (Larsen et al., 2008).

In addition, the augmented activation of inflammatory signals with concurrent PAR-2 activation and LPS exposure has been described (Ostrowska et al., 2007; Chi et al., 2001). In this study, we were interested in investigating whether combinations of the PAR-2 agonist SLIGKV-NH_2 or PAR-2 activating seafood trypsins with LPS could produce enhanced inflammatory signals in the form of NF-κB-activation as well as IL-8

stimulation in our cell model. The combined effects with concomitant exposure to these agents have not been previously described.

The results in Figure 5, reveal an augmented activity of NF-κB in the samples treated with a mixture of LPS and SLIGKV-NH_2 or seafood trypsins compared to those treated with either of the agents alone (Figure 5). A significant 2-fold increase in NF-κB was observed in the cells treated with a mixture of 0.015 mU/ ml king crab trypsin + 10 µg/ml LPS, and a 1.5-fold increase was observed in the cells treated with 0.6 mU/ml salmon trypsin + 10 µg/ml LPS. These results confirm the previous finding that there are differences in the salmon and king crab trypsins regarding the ability of these proteases to generate inflammatory signals (Larsen et al., 2011). The results further indicate that concurrent exposure to LPS and seafood trypsin increases the generation of transcription factor NF-κB compared to exposure to the individual agents, and thus augments the inflammatory signals in the A549 cell line.

Further, to investigate whether the increase in NF-κB signals extents to inflammatory cytokines, we examined the effect on IL-8 of the PAR-2 agonist peptide SLIGKV- NH_2 and seafood trypsins in combination with LPS. We found a significant increase in the IL-8 levels when the A549 cells were treated with mixtures consisting of variable concentrations of the PAR-2 agonist or seafood trypsins with 10 µg/ml LPS (Figures 2, 3A and 3B). With lower concentrations of the seafood trypsins combined with a low concentration of LPS (10 µg/ml) we found that the IL-8 levels after combined exposure were significantly higher than the sum of the levels generated by either of the agents alone. This indicates a synergistic effect of seafood trypsin and LPS at these concentrations. With the higher concentrations of either type of seafood trypsins (Figure 3A and 3B) or LPS (Figure 4), this synergism was no longer evident, indicating that the effect is concentration dependent. These results provide an important new insight into the effects of exposure to mixture of these agents. Therefore, exposure to relatively low concentrations of LPS together with relatively low concentrations of seafood trypsin may cause a considerably augmented inflammatory response in airway cells due to the synergistic interactions between the exposure agents.

Previous studies have demonstrated that the prototype LPS receptor, TLR-4, and the serine protease receptor PAR-2 interact at the level of cross talk between signalling pathways, which augment the inflammatory signals (Gieseler et al., 2013; Rallabhandi et al., 2008). Rallabhandi suggested an interaction between the two hetero-receptors via a MyD88-dependent pathway (Rallabhandi et al., 2008). Receptor cooperativity involving the PAR-2 and TLR-4 receptors may thus offer a likely explanation of the augmented inflammatory signalling observed with concomitant exposure to seafood trypsins and LPS. Further research is required to elucidate the mechanistic details and physiological significance of this interaction.

5. Conclusions

We conclude that bacterial LPS induces the secretion of the inflammatory mediator IL-8 in a dose-dependent manner in the A549 human pulmonary epithelial cell model. We also demonstrate that mixtures of LPS and seafood trypsins have combined effects, augmenting the generation of the transcription factor NF-κB and synergistically enhancing the secretion of IL-8. According to the present findings, our data suggest that exposure to mixtures of environmental LPS and seafood trypsin may elicit an inflammatory response in the airway. This response may vary depending on the seafood species and the concentrations of LPS and proteases in the environment. Further research focusing on the exposure levels of seafood trypsin and endotoxins (LPS) in occupational environments, as well as epidemiological studies, is required to confirm the role of mixed exposures in the generation of airway symptoms in workers of the seafood industry.

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Figures and legends

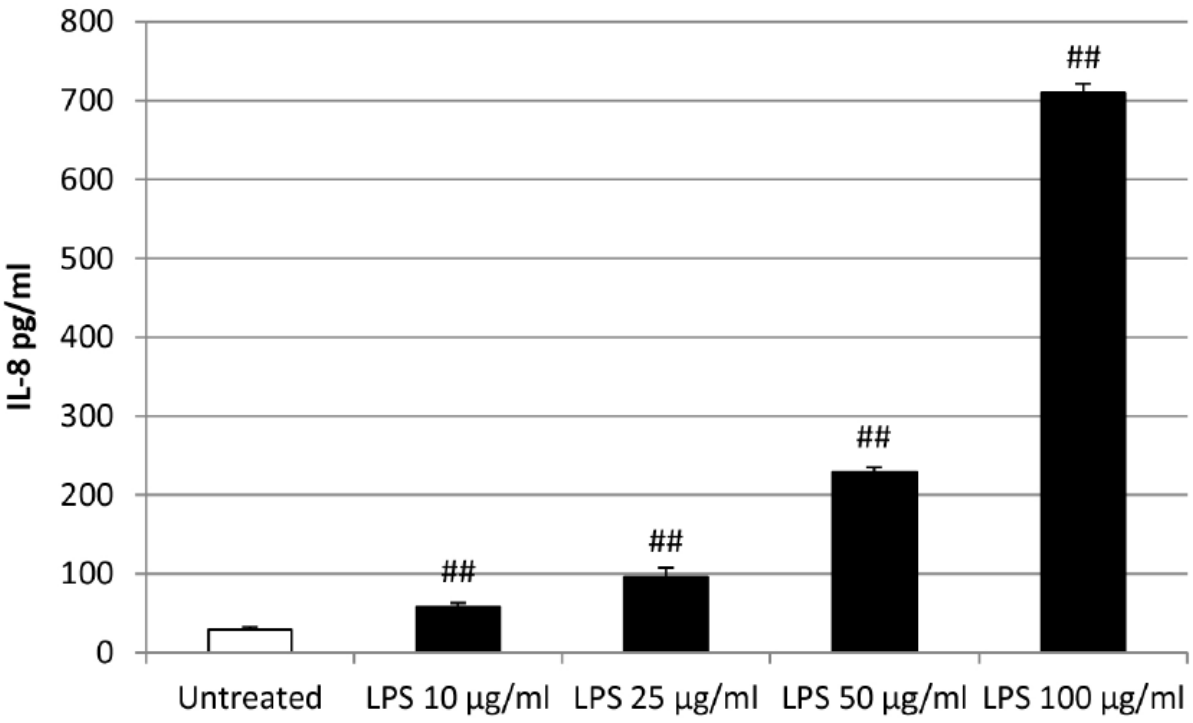

Figure 1. Effect of LPS (endotoxin) on secretion of IL-8 in A549 cells. A549 cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well. After 48 hours, the cells were subjected to starvation in a serum-free medium. Then, 24 hours later, the cells were incubated with the indicated concentrations of LPS for 6 hours. The supernatant was analysed for the presence of IL-8 with an ELISA. The data are presented as the means ± S.D.; n = 6 from three individual experiments. (## $p < 0.01$ compared with untreated cells.)

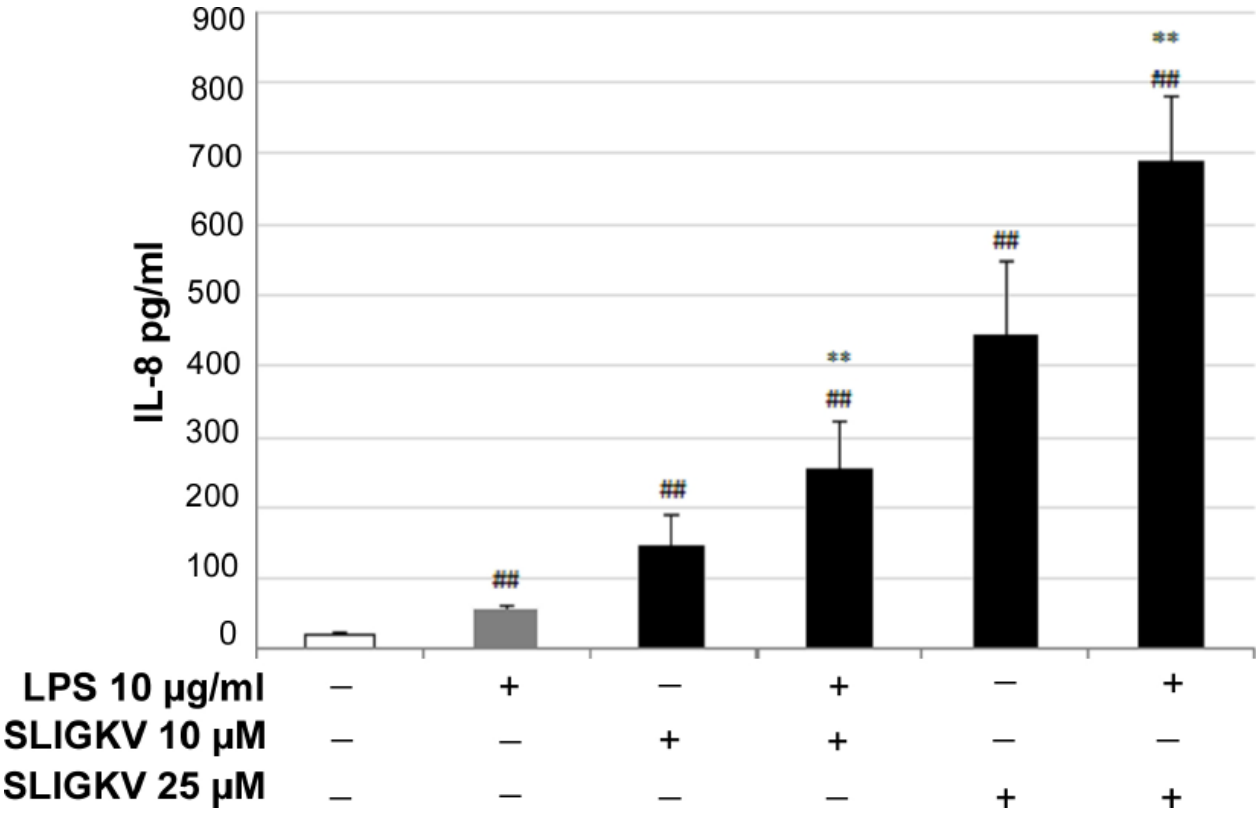

Figure 2. Effect of LPS (endotoxin) and SLIGKV-NH_2 on the secretion of IL-8 in human pulmonary epithelial cells. A549 cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well. After 48 hours, the cells were subjected to starvation in serum-free medium. Then, 24 hours later, the cells were incubated with the indicated concentrations of SLIGKV- NH_2 and LPS for 6 hours. The supernatant was analysed for the presence of IL-8 with an ELISA. The data are presented as the means ± S.D.; n = 6 from three individual experiments. (## $p < 0.01$ compared to the untreated cells; ** $p < 0.01$ compared to the cells treated with SLIGKV- NH_2 alone.)

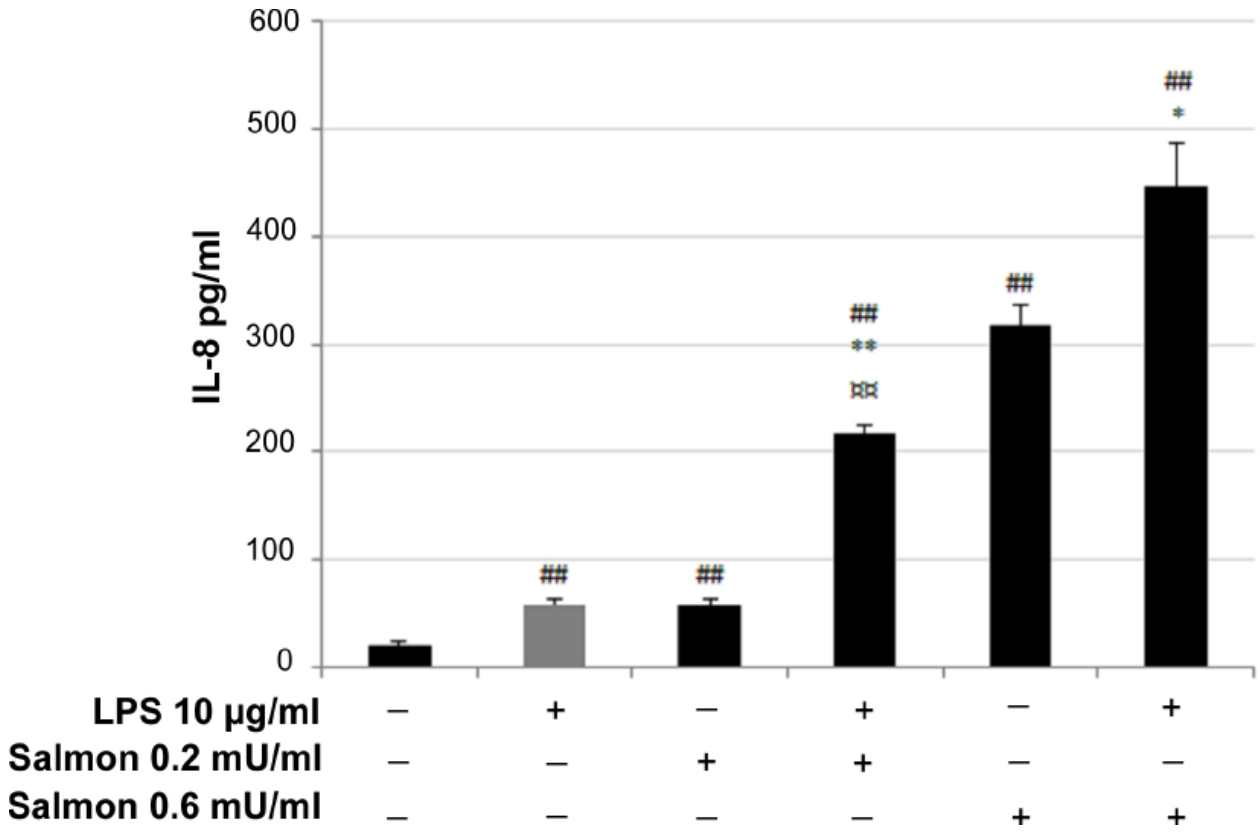

Figure 3A: **Salmon trypsin and LPS synergistically induce secretion of IL-8 in A549 cells.** A549 cells were seeded into 12- well plates at a density of 1 x 10^5 cells/well. After 48 hours, the cells were subjected to starvation in serum-free medium. Then, 24 hours later, the cells were incubated with the indicated concentrations of purified salmon trypsin and LPS (10 µg/ml) for 6 hours. The supernatant was analysed for the presence of IL-8 with an ELISA. The data are presented as the mean ± S.D.; n = 6 from three individual experiments. (## $p < 0.01$ compared to the untreated cells; * $p < 0.05$, ** $p < 0.01$ compared to cells treated with purified salmon trypsin alone; ¤¤ $p < 0.01$ compared to the cells treated with purified salmon trypsin alone + LPS alone.)

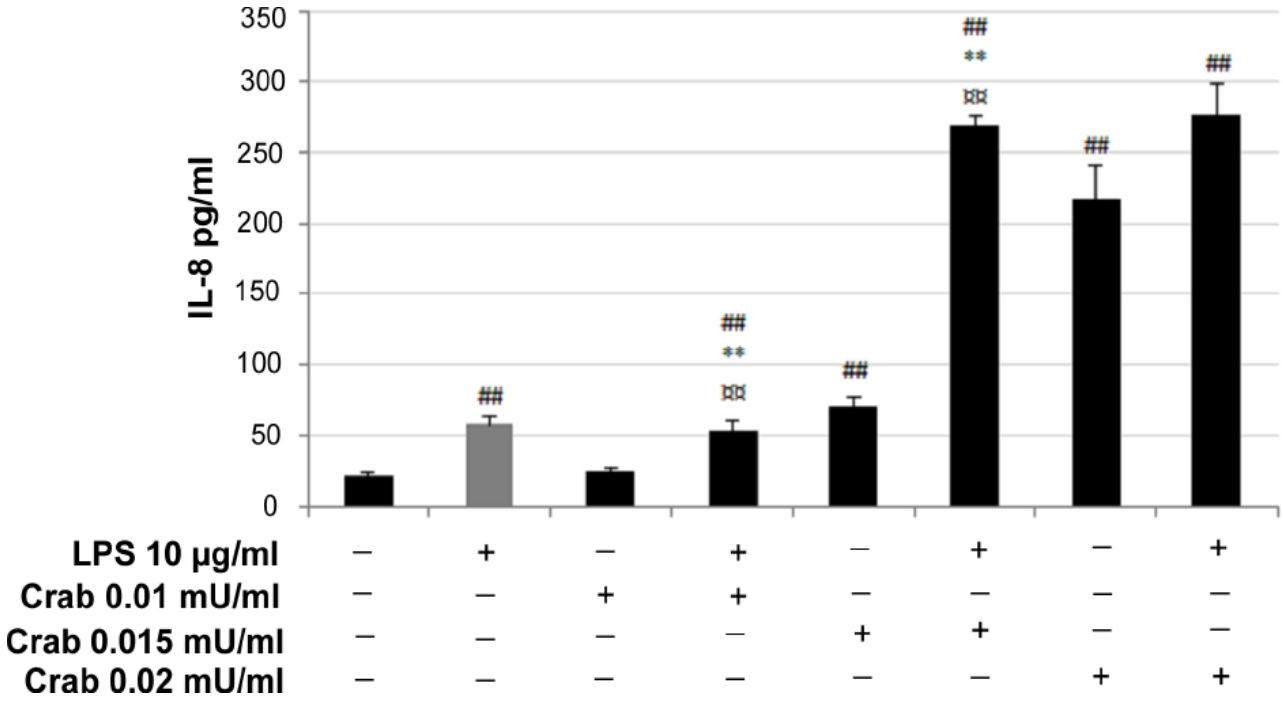

Figure 3B. King crab trypsin and LPS synergistically induce secretion of IL-8 in A549 cells. A549 cells were seeded into 12-well plates at a density of 1 x 10^5 cells/well. After 48 hours, the cells were subjected to starvation in serum-free medium. Then, 24 hours later, the cells were incubated with the indicated concentrations of purified king crab trypsin and LPS (10 µg/ml) for 6 hours. The supernatant was analysed for the presence of IL-8 with an ELISA. The data are presented as the means ± S.D.; n = 6 from three individual experiments. (## $p < 0.01$ compared to the untreated cells,;** $p < 0.01$ compared to the cells treated with purified king crab trypsin alone; ¤¤ $p < 0.01$ compared to the cells treated with purified king crab trypsin alone + LPS alone.)

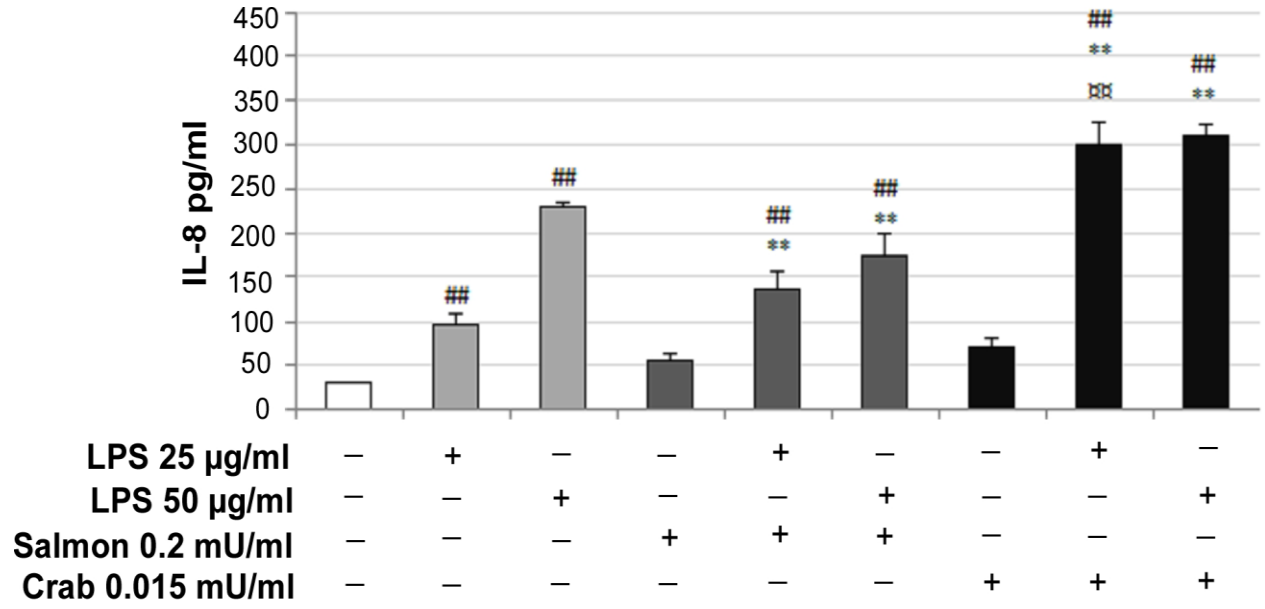

Figure 4. Effect of variable LPS (endotoxin) concentrations. A549 cells were seeded into 12-well plates at a density of 1 x 10^5cells/well and cultured until reaching 80–90% confluency. After 48 hours, the cells were subjected to starvation in serum-free medium for 24 hours, and the cells were then incubated with the indicated concentrations of purified salmon trypsin or king crab trypsin and/or LPS for 6 hours. The supernatants were analysed for the presence of IL-8 with an ELISA. The data are presented as the mean ± S.D.; n = 6 from three individual experiments.(## $p < 0.01$ compared to the untreated cells; ** $p < 0.01$ compared to the cells treated with purified salmon or king crab trypsin alone; ¤¤ $p < 0.01$ compared to the cells treated with purified king crab trypsin alone + LPS alone.)

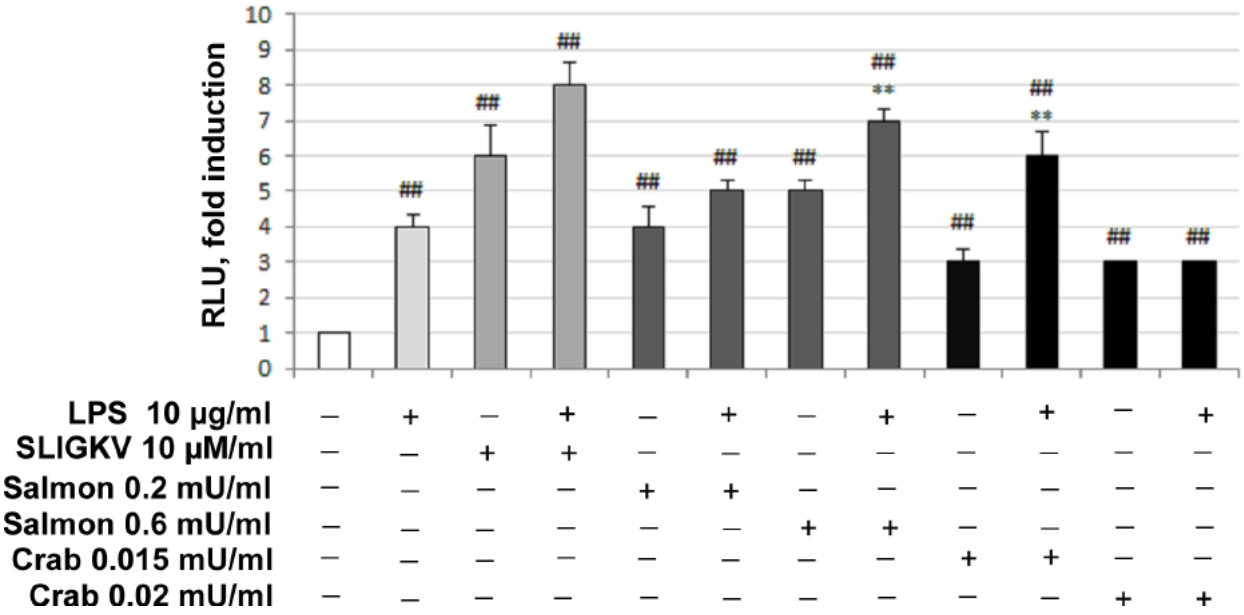

Figure 5. Effect of the mixture of LPS with SLIGKV-NH_2 or the salmon or king crab trypsins on the generation of NF-κB in human pulmonary epithelial cells. A549-NF-κB-luc cells were seeded into 12-well plates at a density of 1 x 10^5 cell/well and grown until the cells were 80-90% confluent. The cells were then subjected to starvation in serum-free medium for 24 hours followed by incubation with the indicated concentrations of the purified salmon or king crab trypsins and/or LPS. After 6 hours, the cells were lysed with a lysis buffer, and the lysates were analysed for luciferase activity. The results were normalised against the total protein concentration and expressed as a fold induction above the basal level. The data are presented as the mean ± S.D.; n = 6 from three individual experiments. (## p < 0.01 compared to the untreated cells; ** p < 0.01 compared to the cells treated with purified salmon or king crab trypsin alone.)