Effect of *Aggregatibacter actinomycetemcomitans* Leukotoxin on ATP Release through Pannexin Channels in Human Monocytes

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ABSTRACT

Aggregatibacter actinomycetemcomitans is strongly associated with aggressive periodontitis and one of several virulence factors is a leukotoxin (LtxA). The toxin has a consequential impact on human leukocytes which leads to an interference with the host’s defences due to a chain reaction involving activation and release of a pro-inflammatory cytokine; interleukin-1β (IL-1β). As an early phase in this reaction chain the toxin stimulates a massive release of adenosine triphosphate (ATP) from human leukocytes. We hypothesize that leukotoxin-induced pro-inflammatory cell death is initiated by ATP release through pannexin channels in the human monocyte cell membrane. The aim of this study was to investigate if blocking of pannexin channels with carbenoxolone (Cbx) results in a reduction of ATP release. A human monocyte cell line (THP-1 cells) was exposed to purified LtxA, Cbx and oxidized ATP. Colorimetric ATP kit was used to evaluate levels of ATP release and thereafter the samples were read in a spectrophotometer. This study confirms that LtxA induces an ATP release from THP-1 cells. Our conclusion is that blocking of pannexin channels does not result in a statistically significant reduction in ATP release, which indicates that ATP is released by one or several other undetected pathways.
INTRODUCTION

*Aggregatibacter actinomycetemcomitans* is a facultative anaerobic, gram-negative coccobacillus. Due to previous longitudinal studies, presence of *A. actinomycetemcomitans* in subgingival plaque can be considered as a risk marker for progression of attachment loss around teeth (Fine *et al*., 2007; Haubek *et al*., 2008). The bacterium is associated with periodontal bone loss, carrying the bacterium in the oral cavity however does not necessarily lead to development of periodontitis (Fine *et al*., 2007; Höglund Åberg *et al*., 2014a). *A. actinomycetemcomitans* is strongly associated with aggressive forms of periodontitis, especially local aggressive periodontitis (LAP) in young individuals (Haubek *et al*., 2008; Slots & Ting, 1999).

The pathology of *A. actinomycetemcomitans* is a result of a complex bacterium-host interrelationship (Henderson *et al*., 2010; Slots & Ting, 1999). The bacterium has several virulence factors that facilitates colonization, invasion, immunosuppression and immunoevasion. Furthermore, it interferes with tissue remodelling resulting in destruction (Henderson *et al*., 2010). Among these virulence factors there are two exotoxins where one of them is the leukotoxin; a large pore forming protein that belongs to the Repeats in Toxin (RTX) family (Lally *et al*., 1999). The toxin can be found on the bacterial surface as well as it can be released under certain circumstances from *A. actinomycetemcomitans*’ outer membrane and vesicles (Johansson *et al*., 2003). This toxin has shown to be very important in the pathogenicity because of the consequential impact on human leukocytes, which leads to an interference with the host’s defences (Johansson, 2011). All strains of *A. actinomycetemcomitans* have a complete leukotoxin coding operon, but their expression strongly varies due to a great genetic diversity that results in genotypes with different capacity for leukotoxin production; leukotoxicity (Haubek *et al*., 2007; Höglund Åberg *et al*., 2014a). The operon consists of four genes; *ltxC, ltxA, ltxB* and *ltxD* in combination with a promoter. The variation in leukotoxicity depends on the different strains’ ability to express these genes (Johansson, 2011). Leukotoxin A (*LtxA*) is the gene product of the coding gene *ltxA* in the operon (Fine *et al*., 2006). *ltxC* stands for posttranslational
modification that is required for the leukotoxin to be biologically active (Lally et al., 1999). The ltxB och ltxD genes enable toxin transport to the bacterial outer membrane (Johansson, 2011). The risk of developing aggressive periodontitis is enhanced in individuals carrying highly leukotoxic bacteria (Höglund Åberg et al., 2014b). The JP2-clone is a highly leukotoxic and virulent genotype that has been described in earlier studies to be strongly coupled with more aggressive forms of periodontitis (Haubek et al., 2008; Höglund Åberg et al., 2014a). It has been suggested that increased leukotoxicity could be explained by an expression due to modification in the promoter region on the leukotoxin producing operon (Brogan et al., 1994).

LtxA has its own specificity for human immune cells due to electrostatic forces between the ltxA-repeat region and the target cell receptor on the plasma membrane; lymphocyte function-associated antigen-1 (LFA-1) (Lally et al., 1997). When LtxA is attached to LFA-1, a massive release of adenosine triphosphate (ATP) occurs from within the immune cell (Kelk et al., 2011). ATP release activates a purinergic P2X7-receptor located on the cell surface of human monocytes (Ferrari et al., 1997). This results in an efflux of potassium (K+) that leads to intracellular caspase-1 inflammasome activation and release of interleukin-1β (IL-1β) (Ferrari et al., 2006). These inflammasomes are activated in several different pathways where extracellular ATP has been identified as one activator (Bergsbaken et al., 2009; Yu & Finlay, 2008). IL-1β is a pro-inflammatory cytokine with the ability to activate bone-resorbing cells; osteoclasts, that are essential in the pathology of periodontitis (Dewhirst et al., 1985; Lorenzo et al., 1987). Activation of caspase-1 can induce a specific kind of cell death called pyroptosis. Pyroptosis implicates development of pores in the cell membrane. These pores cause increased osmotic pressure and an accumulation of water that leads to swollen cells and eventually osmotic cell lysis as well as release of pro-inflammatory intracellular content (Bergsbaken et al., 2009). LtxA seems to induce a specific inflammatory cell death in human immune cells very similar to pyroptosis (Kelk et al., 2011).
Presence of *A. actinomyctemcomitans* and LtxA activates the human immune system resulting in a recruitment and differentiation of human monocytes into macrophages in the infected tissue (Fine *et al.*, 2006). The human cells used in this study can be differentiated into macrophages and derive from a human leukemia monocytic cell line (THP-1 cells), commonly used in scientific studies (Tsuchiya *et al.*, 1980). Release of extracellular lactate dehydrogenase (LDH) is a known measurement of cell injury and membrane leakage that can give an indication of LtxA-induced cell lysis (Kelk *et al.*, 2003). LDH catalyze the oxidation of NADH to NAD, and cause a decrease in absorbance (Wroblewski & Ladue, 1955).

The focus in this project will be leukotoxin-induced ATP release and the involvement of pannexin channels and purinergic receptors in this process. Pannexins are a family of transmembrane proteins (Panx1, Panx2 and Panx3) (Baranova *et al.*, 2004; Panchin *et al.*, 2000). These hemichannel-forming proteins have qualities similar with the well-investigated connexin gap junction proteins (Penuela *et al.*, 2007; Penuela *et al.*, 2009). Both connexins and pannexins have intracellular amino- and carboxy-terminals and two extracellular loops with regularly spaced cysteine residues; three in connexins and two in pannexins (Barbe *et al.*, 2006). Previous studies have shown that Panx1 allows ATP efflux by channel-opening when depolarized by elevated extracellular potassium levels (Bao *et al.*, 2004). Carbenoxolone (Cbx), a non-selective gap junction channel blocker, has been proven to be an effective inhibitor of Panx1 currents (Ma *et al.*, 2009).

We have previously shown that inhibition with oxidized ATP (oATP) of the purinergic receptor P2X7 completely blocks the effect of leukotoxin on human macrophages (Kelk *et al.*, 2011). This indicates that the leukotoxin-induced macrophage activation involves release of intracellular ATP. Regarding this we propose following hypothesis: Leukotoxin-induced pro-inflammatory cell death is initiated by ATP release through pannexin channels. The aim of this study was to investigate if blocking of pannexin channels with carbenoxolone (Cbx) results in a reduction of ATP release.
MATERIALS & METHODS

Literature
A literature search was made on PubMed, using several MeSH-terms in different combinations. Following terms were used regarding *A. actinomycetemcomitans* involvement in periodontal disease: ("aggregatibacter actinomycetemcomitans"[MeSH Terms] AND "virulence"[MeSH Terms]) OR "aggregatibacter actinomycetemcomitans"[MeSH Terms]) AND "exotoxins"[MeSH Terms].

Concerning more detailed molecular mechanisms other MeSH-terms had to be used: (connexin/genetics[MeSH Terms]) OR gap junctions) AND glycosylation[MeSH Terms], (Connexins/genetics*[MeSH Terms]) AND Connexins/physiology*[MeSH Terms]) AND Pannexin) AND connexin, ("adenosine triphosphate"[MeSH Terms] AND interleukin 1/metabolism[MeSH Terms]) AND receptors, purinergic p2/metabolism[MeSH Terms]) AND "interleukin-1/secretion"[Mesh Terms]. Selected articles were also recommended to us by our tutors.

Ethical consideration
This is an experimental study and it does not involve any material from test individuals. The commercially available THP-1 cell line cannot be traced and is widely used in scientific studies. The possible outcome of this study may contribute to a wider understanding of the specific mechanisms associated with LAP that in the future can conduce to development of new therapeutic drugs. Good scientific praxis was applied concerning all experimental data processed in this study.

Cell culture
A human monocyte cell line THP-1 (TIB202™, ATCC®, Manassas, USA) was used in all laboratory experiments performed in this study. The cells were quantified with a Bürker chamber and a cell concentration of 10^6 cells/mL was prepared with RPMI-1640 10 % fetal bovine serum (FBS) (Gibco®, Paisley, Scotland) and
differentiated with 50 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich®, Saint Louis, USA). After incubated for 24 hr in 37 °C the medium was replaced with RPMI-1640 10 % FBS and again incubated for another 24 hr. The medium was substituted again before conducting experiments.

**Purification of leukotoxin**

The LtxA used in this study was purified from the strain HK 1519 of *A. actinomycetemcomitans*. Gel filtration chromatography was used to purify leukotoxin from the crude extract after elimination of other membrane proteins than LtxA (Johansson *et al.*, 2000).

**LDH release**

Measurement of LDH release is a simple method for determination of cell injury and membrane leakage. In order to evaluate the capacity of LtxA-induced cell injury the LDH release from THP-1-cells was measured. This enabled determination of proper concentrations of LtxA to use in nextcoming laborations.

The cells were prepared as earlier described and the medium was substituted with 100 μL RPMI-1640 0 % FBS. A stock solution of 360 μg/mL purified LtxA was diluted with RPMI-1640 to get 200 ng/mL. An amount of 100 μL 200 ng/mL LtxA was then added to the cells and a serial dilution was performed. A volume of 200 μL RPMI-1640 was prepared as a negative control and 200 μL 0.1 % Triton as a positive control. Triton solution is a non-ionic detergent that can cause total cell lysis and can therefore be used to determinate maximum LDH release (Koley and Bard, 2010). The plate was then incubated for 1 hr in 37 °C. Phosphate buffer was prepared with 305 mL 0.1 M Na₂HPO₄ and 195 mL 0.1 M NaH₂PO₄ under gently stirring until pH 7 was reached. Substrate buffer was prepared with 28 mL 0.1 M phosphate-buffer (pH 7) mixed with 1 mL freshly prepared 2.5 mg/mL Na-pyruvate and 5 mg/mL NADH-Na. After incubation, samples of 25 μL were transferred into a new 96-welled microtiter plate and 180 μL substrate buffer was added. The absorbance was continuously read at 340 nm with a spectrophotometer during 5 min and a decrease in absorbance was expected due
to LDH catalyzed oxidation of NADH to NAD. The decrease in absorbance was calculated and expressed in relation to the triton lysed cells (100 %).

**LtxA-induced ATP release**

It is known that LtxA induces an ATP release from human monocytes. The ATP release was measured over time with cells exposed to different concentrations of LtxA.

A stock solution of purified LtxA (360 μg/mL) was diluted with RPMI-1640 into three solutions: 200 ng/mL, 20 ng/mL and 2 ng/mL. To evaluate the effect on ATP release from PMA differentiated THP-1 cells, 500 μL of each LtxA solution was added separately. The cell medium was replaced with 500 μL of RPMI-1640. RPMI-1640 was also used solitary as a negative control and a positive control was seen as the ATP release caused by LtxA 100 ng/mL at 30 min (100 %). The plate was then incubated in 37 °C and 50 μL samples were transferred after 1, 10 and 30 min into separate Eppendorf tubes and then put on ice for later analysis. ATP was measured with an ATP Assay Kit (ab83355) (abcam®, Cambridge, England) where phosphorylation of glycerol is utilized to create a product that can be quantified by colorimetric methods (abcam®). The kit consisted of ATP assay buffer, ATP Converter, ATP Probe, ATP Standard and Developer Mix, prepared according to the manufacturer’s instructions. A standard was serial diluted and a reaction mixture was prepared and 50 μL was added in every well. The samples of 50 μL that were put on ice were transferred into the new wells. The plate was incubated protected from light for 30 min in room temperature and the absorbance at 570 nm was subsequently read with a spectrophotometer.

**Effect of inhibitors of LtxA-induced ATP release**

The PMA differentiated THP-1 cells were prepared to determinate the effect of pannexin channel inhibition with Cbx (Sigma-Aldrich®, Saint Louis, USA). RPMI-1640 and 10 mM Cbx were diluted to 200 μM and thereafter RPMI-1640 and 10 mM oATP (Sigma-Aldrich®, Saint Louis, USA) were diluted to the same concentration. The cell culture medium was replaced with 250 μL RPMI-1640 0 %
FBS, 250 μL 200 μM Cbx and 250 μL 200 μM oATP separately in duplicates. The plate was then incubated in 37 °C and a solution of 200 ng/mL LtxA with RPMI-1640 was prepared. A volume of 250 μL RPMI-1640 was added to one of each duplicates and 250 μL 200 ng/mL LtxA was added to the other duplicates. The plate was then incubated for 30 min in 37 °C. The ATP Abcam kit was used to analyse the ATP release and the absorbance was read at 570 nm with a spectrophotometer. This experiment was repeated four times.

Statistical analyses
Mean and standard deviation was calculated in Microsoft office Excel and student t-test was used to calculate significant differences. Significant differences was indicated when p values were ≤ 0.05.

RESULTS
LDH release
The LtxA purified from A. actinomycetemcomitans caused a dose-dependent increased cell lysis measured as the activity of extracellular release of cytosolic LDH. An increase in LDH is seen with higher LtxA concentrations reaching 37.7 % of maximum cell lysis after 5 min at 100 ng/mL LtxA (Fig. 1).

LtxA-induced ATP release
LtxA cased a time- and dose-dependent release of ATP from cultures of PMA-differentiated THP-1 cells. LtxA 100 ng/mL shows a much greater increase in ATP release than the lower concentrations, compared with negative control (Fig. 2).

Effect of inhibitors of LtxA-induced ATP release
A significant increase in ATP release was shown between the negative and positive control (p value 0.001). The increase in ATP release was also significant in the solution containing Cbx and LtxA (p value 0.019) as well as oATP and LtxA (p value 0.019) compared to the negative control. Cbx inhibition of pannexin channels revealed a slight but not statistically significant reduction in ATP release compared
to the positive control (p value 0.248). Inhibition of the P2X<sub>7</sub> receptor with oATP showed no decrease in ATP release (p value 0.148) (Fig 3).

**DISCUSSION**

No significant reduction of LtxA-induced ATP release was obtained by blocking of pannexin channels with Cbx. This indicates that one or several other undetected pathways releases ATP.

LDH is known to be an indicator of cell lysis and earlier studies have shown that leukotoxin-induced cell lysis successfully can be measured (Wroblewski and LaDue, 1955; Kelk *et al*., 2003). Measurement of LDH from LtxA-exposed THP-1 cells in this study confirms a great potency for leukotoxin-induced cell lysis. The increase in LDH release with increasing concentrations of LtxA shows the greatest percentage of cell lysis at the concentration 100 ng/mL LtxA, representing 37.7% of the positive control. We decided that 100 ng/mL was a suitable concentration to use in next coming experiments, due to the intermediate effect on LDH release, which both can be decreased or increased.

It is known that LtxA induces a massive ATP release from THP-1 cells (Kelk *et al*., 2011). The concentration 100 ng/mL LtxA shows sufficient ATP increase compared with negative control and is therefore appropriate for measuring ATP release from THP-1 cells. The lower LtxA concentrations as well as shorter incubation times display values too close to the negative control. In the final experiment a significant increase in ATP release is seen in all test groups compared with the negative control. We have previously shown that oATP inhibition of the purinergic receptor P2X<sub>7</sub> completely blocks the effect of leukotoxin on human macrophages (Kelk *et al*., 2011). The blocking of this receptor inhibits the effect of ATP after extracellular release and is therefore not expected to affect the LtxA-induced ATP release, which we confirm in this study. It can therefore be assumed that oATP could be a potential drug for treatment of *A. actinomycetemcomitans*-associated periodontitis but further studies are needed. Cbx is a known non-selective inhibitor of pannexin channels (Ma *et al*., 2009). Our hypothesis was that
ATP is released through pannexin channels (Panx1) in LtxA-exposed THP-1 cells and that addition of Cbx would result in a reduction of extracellular released ATP. Our statistical data from student t-test does not confirm this hypothesis. Mean values from the four experiments show a small, but not significant reduction of ATP release.

Previous studies have shown that Panx1 allows ATP efflux and Cbx has been proven to be an effective inhibitor of the Panx1 currents (Bao et al., 2004; Ma et al., 2009). ATP release is an early stage in the inflammatory response that can lead to periodontitis. If a significant blocking of ATP was achieved, it would indicate that Cbx could be a potential therapeutic drug in treatment of A. actinomycetemcomitans-associated periodontal disease. If blocking can be established, the secretion of the pro-inflammatory cytokine IL-1β will be inhibited which results in a lesser amount of activated osteoclasts; bone-resorbing cells that are essential in the pathology of periodontitis (Dewhirst et al., 1985; Lorenzo et al., 1987). The treatment methods of today are primitive and focused on removal of periodontal pathogens by teeth debridement and instructions in oral hygiene. Many years of scientific research has lead to a wide understanding of mechanisms regarding the pathogenicity of periodontitis. This provides possibilities for development of more effective and precise molecular biologic treatments, an interesting field for further research.

CONCLUSION
This study confirms that LtxA induces a release of LDH and ATP from PMA-differentiated THP-1 cells. Blocking of pannexin channels show no significant reduction in LtxA-induced ATP release, which indicates that ATP is released by one or several other undetected pathways. For deeper understanding of the mechanisms regarding ATP release, further studies are needed.

ACKNOWLEDGEMENTS
We wish to acknowledge the appreciated guidance provided by our main supervisor Anders Johansson and our second supervisor Peyman Kelk.
REFERENCES


Fig. 1: Increasing THP-1 cell lysis with higher concentrations of LtxA. PMA-differentiated THP-1 cells were exposed to LtxA from *A. actinomycetemcomitans* for 60 min and cell lysis was measured as the activity of released LDH from damaged cells. Cell lysis is expressed in relation to maximum release from triton-lysed cells (100 %), used as a positive control. Mean ± SD from triplicates.
Fig. 2: Time- and dose-dependent release of ATP from THP-1 cells upon exposure to LtxA. The PMA-differentiated THP-1 cells was exposed to different concentrations of LtxA and the released ATP was examined at 1, 10, 30 min. The ATP release is expressed in relation to that caused of LtxA 100 ng/mL at 30 min (100 %).
Fig. 3. Effect of Cbx or oATP on THP-1 cells exposed to LtxA (100 ng/mL) for 30 min. The release of ATP is expressed in relation to that caused by 30 min of LtxA (100 ng/mL) exposure (100 %). Mean ± SD from four experiments are shown and significant differences from the control cells are indicated with *.