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Characterizing ATP-dependent activation of inflammasome in Gingival Epithelial Cells

in

Quantitative and Systems Biology

by

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Committee in charge:

Professor David M. Ojcius, Chair
Professor Jinah Choi
Professor Ajay Gopinathan

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DEDICATION

I would like to dedicate my thesis to the lab members of the Ojcius lab (2009-2010) as well as the undergrads working there. Without their guidance and support through this year, this piece of work may never have come to be.

I would also like to extend my thanks to my friend Malak Hahris without who's help; I may not have survived the year.

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ABSTRACT OF THE THESIS

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The release of the pro-inflammatory cytokine IL-1β from an epithelial cell is dependent on two separate signals. The first signal comes from the binding of a pathogen

associated molecular pattern to a pathogen recognition receptor, which then promotes the production of the immature form of IL-1β. The second signal will come from a danger signal in the form of extracellular ATP, which will lead to the assembly of an inflammasome, activation and caspase-1 and secretion of the mature IL-1β. This thesis evaluates whether the mechanism by which extracellular ATP activates the inflammasome is in a P2X7 dependent manner. The characterizing of this mechanism is to be done for the first time in gingival epithelial cells (GECs), the first host cells a periodontal pathogen like *Porphyromonas gingivalis* would encounter. We have shown that GECs express a functional P2X7 receptor, but the mechanism by which we get activated caspase-1 is still uncertain. In this study, we compared reactive oxygen species (ROS) production levels between cells treated with just ATP and cells pretreated with a P2X7 antagonist. The cells treated with the antagonist before the ATP showed significantly lower levels of ROS production suggesting that ATP stimulated ROS production is P2X7 dependent. ROS production has been shown to promote the assembly of the inflammasome, leading to caspase-1 activation. Thus, we also want to show that caspase-1 activation by ATP stimulation is P2X7 dependent. Our results on that front are still inconclusive. After this project, we still need to investigate the effects of infection by *P. gingvalis* and its NDK deficient mutant on ROS production and caspase-1 activation.

INTRODUCTION

Gingival epithelial cells (GECs) are the first host cells that periodontal pathogens such as *Porphyromonas gingivalis* encounter upon entrance into the gingival compartment. These epithelial cells are not only a physical barrier to invasion, but they can also sense and respond to the presence of bacteria through the stimulation of their pattern recognition receptors (PRRs) and NOD-like receptors (NLRs)[1]. *P. gingivalis* is a gram-negative bacteria that preferentially infects GECs. It is commensal, but could become pathogenic and is considered an etiological agent in severe forms of adult periodontitis. Periodontitis is the inflammation of the periodontium which produces bleeding, pus formation, and a gradual loss of the bone and tissue that support the teeth.

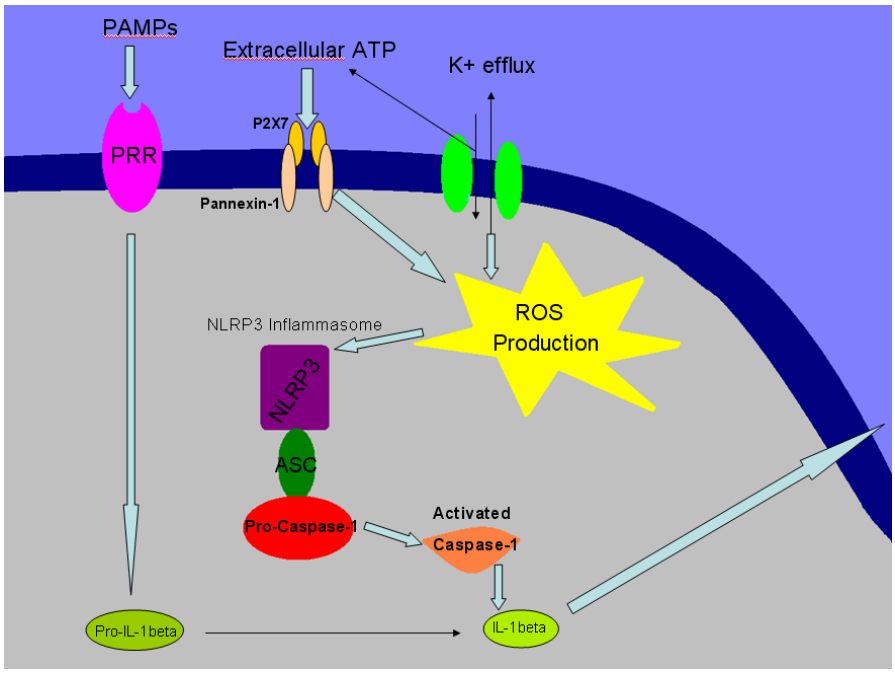

Diagram 1 Proposed inflammasome mechanism

Thus, GECs are the crucial first line of defense in the innate immune response of the host to periodontal pathogens making it an important part of the host's gingival health[2].

The ligation of pathogen associated molecular pattern (PAMP)s, derived from the invading organism, to a PRR is enough to commence the transcription and synthesis of

the pro-inflammatory cytokine, pro-IL-1β. However, a second, 'danger signal' (DS), is needed for the processing and secretion of its mature form, IL-1β[3]. This DS could come from invading pathogen, or host-cell material that was released from dying, infected, or stressed cells, like ATP[4]. It then promotes the assembly of the multi-protein complex known as the 'inflammasome' in the cytoplasm of the cell. This complex is made up of a molecule of the NLR family and ASC (apoptosis-associated speck-like protein containing a caspase activation recruitment domain), an adaptor protein[5]. The 'inflammasome' leads to the activation of caspase-1, which would then cleave the pro-IL-1β into IL-1β and lead to its secretion (refer to Diagram 1).

The DS that we are exploring in this thesis is extracellular ATP and by what mechanism it follows to the activation of caspase-1. The P2X receptors are a family of ligand-gated ion channels whose ligand is extracellular ATP. More specifically, ATP binds to the P2X7 receptor, known to be expressed in macrophages and some epithelial cells[6]. And depending on cell type, the ligation of P2X7 can lead to apoptosis, cell proliferation, or the killing of intracellular pathogens. The binding of ATP to a P2X receptor results in a conformational change in the ion channel that opens the channel, letting Na^+ and Ca^{2+} into the cell. This causes a depolarization of the cell membrane, and the subsequent efflux of K^+. This in turn leads to the assembly of the NLRP3 inflammasome, caspase-1 activation, and IL-1β processing and secretion[7]. Pannexin-1 has been demonstrated to be necessary for ATP-P2X7 dependent caspase-1 activation followed by mature IL-1β release although the specific mechanism is still unknown[8]. The ligation of the P2X7 receptor with ATP has also been shown to cause the formation of reactive oxygen species (ROS) consequently leading to caspase-1 activation in

macrophages[9]. These inflammasome mechanisms have been characterized in monocytes, macrophages, as well as HeLa cells, but never in GECs.

Previous research has shown that ATP stimulates the assembly of the NLRP3 inflammasome in GECs. In the GECs that were NLRP3 deficient, ATP was unable to stimulate IL-1β secretion from infected GECs, while in contrast, GECs that were not NLRP3 knocked down showed a significantly greater amount of IL-1β secretion[6]. Also in GECs, ATP has been shown to induce apoptosis, but blocking the P2X7 receptor using an irreversible antagonist, oxidized (ox)ATP inhibits the ATP-induced apoptosis. Conversely, using a P2X7 receptor agonist, 2',3'-O-(4-benzoyl-benzoyl)ATP (bzATP), to stimulate the receptor induces apoptosis, suggesting that the ATP-induced apoptosis is P2X7 dependent[7].

While ATP may induce apoptosis, there is evidence showing that *P. gingivalis* infection inhibits ATP-induced apoptosis. This is accomplished by the nucleoside diphosphate kinase (NDK) protein encoded in the *P. gingivalis* genome. NDK is an ATP-scavenging enzyme that when knocked down in infecting *P. gingivalis*, followed by a treatment of ATP actually seems to increase apoptosis. But when a complementary copy of the gene was inserted into the infecting *P. gingivalis*, there was again a decrease in apoptosis, even with an ATP treatment, thus giving insight into how *P. gingivalis* is capable of maintaining itself intracellularly for an extended amount of time. Further studies showed that *P. gingivalis* infection alone was not enough to stimulate caspase-1 activation in GECs. It required co-stimulation with ATP for there to be a significant amount of caspase-1 activation[7].

Given that we have observed that ATP works as a danger signal leading up to the processing and secretion of IL-1β, and the fact that it activates caspase-1 in a P2X7 dependent manner, we want to show that the effects of ATP in GECs are solely from a P2X7 dependent manner. Also, because ATP binding to P2X7 appears to lead to inflammasome activation through multiple pathways, we'd like to explore further the mechanism by which the inflammasome is activated.

EXPERIMENTAL PROCEDURES

Cells and Chemical Reagents

Gingival epithelial cells, obtained from the Ozlem laboratory (Gainesville, FL), were cultured in a humidified incubator at 37ºC with 5% CO_2 in Defined Keratinocyte Serum Free Medium (K-SFM) (Invitrogen) supplemented with K-SFM growth supplement (Invitrogen), Bovine Pituitary Extract (Invitrogen), and 10 μg/ml gentamicin (Omega Scientific, Trazana, Ca). Adenosine-triphosphate (ATP), Adenosine-diphosphate (ADP), Adenosine-monophosphate (AMP), Adenosine (ADO), Uridine-triphosphate (UTP), oxidized Adenosine-triphosphate (oxATP), Nigericin, and Probenecid were all obtained from Sigma (St. Louis, MO).

Cell Culture and Treatment

GECs were grown to 60-70% confluence in tissue culture plates (Costar), media was changed a day pre-treatment, and incubated at 37ºC in 5% CO_2 during, in-between treatments, and during dye loading. Treatments with reagents were performed at the indicated times and concentrations.

RNA Extraction

Total RNA was extracted from the GECs using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The total RNA was quantified by measuring the optical density with the NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

cDNA Synthesis, PCR Amplification

After extraction, the RNA for the purinergic receptor genes were converted into cDNA by reverse-transcription, and amplified using the Qiagen Fast Cycling PCR kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The primers for the human GAPDH, the housekeeping gene control, were 5'-cgaccactttgtcaagctca-3' forward and 5'-aggggagattcagtgtggtg-3' reverse. Primers for the human P2X4 receptor were 5'-GATACCAGCTCAGGAGGAAAAC-3' forward and 5'-GCATCATAAATGCACGACTTGAG-3' reverse. Primers for the human P2X7 receptor were 5'-TGATAAAAGTCTTCGGGATCCGT-3' forward and 5'-TGGACAAATCTGTGAAGTCCATC-3' reverse. Primers for the human P2Y1 receptor were 5'-CTTGGTGCTGATTCTGGGCTG-3' forward and 5'-GCTCGGGAGAGTCTCCTTCTG-3' reverse. Primers for the human P2Y11 receptor were 5'-GAGGCCTGCATCAAGTGTCTG-3' forward and 5'-ACGTTGAGCACCCGCATGATG-3' reverse. Primers for the human P2Y13 receptor were 5'-GGAAGCAACACCATCGTCTGTG-3' forward and 5'-GACTGTGAGTATATGGAACTCTG-3' reverse. Primers for the human Pannexin-1 receptor were 5'-GGTGAGACAAGACCCAGAGC-3' forward and 5'-GGCATCGGACCTTAACACCTA-3' reverse. The Fast Cycling PCR protocol for all the primers was a 5 min enzyme activation step at 95ºC, then 45 cycles of 96ºC for 5 seconds, 55ºC for 5 seconds, and 68ºC for 20 seconds. At the end, there was a 10 min extension step at 72ºC.

In order to check whether the GECs expressed the $P2X_7$, $P2X_4$, $P2Y_1$, $P2Y_{11}$, $P2Y_{13}$, and Pannexin-1 receptor genes, after the PCR amplification for those specific

human genes was carried out, the products were separated by electrophoresis on a 2% agarose gel. The gel was run at 100V for 45 min. Then it was visualized by ethidium bromide staining on a gel doc system (Bio-Rad).

Fluorescent Microscopy

Following indicated treatments, supernatant was aspirated from treatment plates and each well was washed twice with warm PBS prior to loading the ROS indicator dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate,acetylester (CM-H_2DCFDA; DCF for short), reconstituted per the manufacturer's suggestion. The treated cells were incubated with 2.5μM DCF for 15 minutes, the plate covered with foil to avoid exposure. 5 minutes prior to the end of the DCF incubation, Hoechst stain (from FLICA^{TM} Caspase-1 Detection kit at 200μg/mL, ImmunoChemistry Technologies) was loaded 2.5μL/500μL. Once stain incubation is done, each well is washed 4 times for 2 minutes each time, covered with foil, and swirled to wash out background staining. Then each well was loaded with phenol red-free Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. ROS production was image captured on a widefield fluorescence microscope (Leica, Deerfield, IL), ROS production (DCF staining) captured at 350ms exposure, and nucleus (Hoechst staining) captured at 110ms exposure.

Quantification of Fluorescent Intensity

The production of ROS with respect to the number of cells was quantified by measuring the intensity of the fluorescence emitted from the acquired images with NIH

ImageJ analysis software. Using the freehand selection tool, each individual cell was selected in the image field, and then the RGB was measured based on pixel intensity. In order to determine the relative fluorescence intensity, the ratio of the mean intensities of green (DCF) to blue (Hoechst) were taken in each field, then that ratio was averaged with consideration to the number of cells total over the fields (that were circled for measurement). The results were then expressed as the fold decrease in fluorescent intensity compared to the positive control, ATP.

Western Blotting

Samples were lysed using RIPA Lysis Buffer (Millipore), protein concentrations were evaluated by the Bradford assay and loaded into a 12% SDS-polyacrylamide gel, then transferred to a polyvinylidene difluoride membrane (Millipore). The blots were blocked for 1 h in 5% (w/v) nonfat dried milk in TBST. The membrane was incubated overnight at 4ºC with rabbit anti-human caspase-1 antibody (Millipore) and then incubated again with HRP conjugated goat anti-rabbit IgG antibody (Millipore). The proteins were detected with ECL Plus Western Blotting Detection Reagents (Amersham) using a gel doc system (Bio-Rad).

FLICA Staining

After treatment, cells were detached using TrypLE^{TM} Express (Invitrogen) and then incubated with 1x FLICA for 1 hr, in which the cells are labeled with FAM-YVAD-fmk caspase-1 FLICA kit (Immunochemistry, Bloomington, IN) that binds activated caspase-1. Incubation was followed by two washes, and flow cytometric analysis was

performed following the manufacturer's manual using the GUAVA EasyCyte (Guava technologies, Hayward, CA).

RESULTS

GECs express the common purinergic receptors (P2X7)

We first checked to see if the GECs expressed common purinergic receptors. The mRNA was extracted from untreated GECs then we used PCR amplification with the primers specific for the human P2X4 (amplicon expected size, 393 bp), P2X7 (amplicon expected size, 396 bp), P2Y1 (amplicon expected size, 327 bp), P2Y11 (amplicon expected size, 260 bp), P2Y13 (amplicon expected size, 293 bp), and Pannexin (amplicon expected size, 199 bp) receptors. The cDNA was then run on a PCR gel with GAPDH as the housekeeping gene control. We found that the GECs definitely express the P2X7, P2Y1 and Pannexin-1 receptors (Fig 1). The P2X4 and P2Y13 receptor mRNAs were also detectable although to a lesser extent, and while there was a visible band found in the P2Y11 lane, it didn't correspond to the expected size.

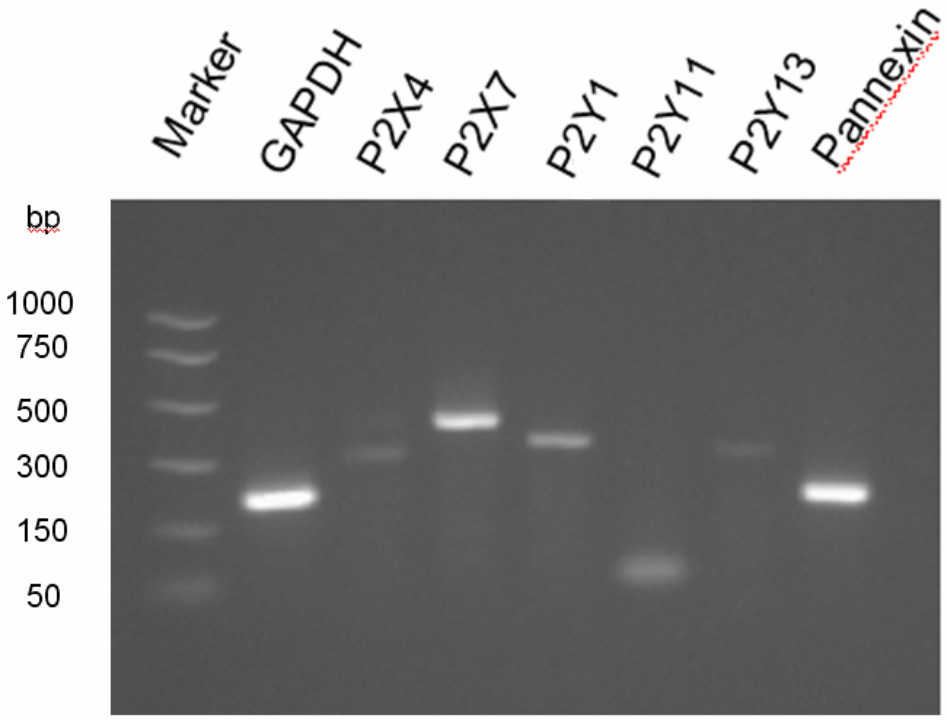

Fig. 1 PCR amplification was carried out with primers specific for P2X4, P2X7, P2Y1, P2Y11, P2Y13, and Pannexin-1 receptors as described in the *Experimental procedures*. Amplicons of the expected sizes were found for P2X4 (393bp), P2X7 (396bp), P2Y1 (327bp), P2Y13 (293bp), and Pannexin-1 (199bp), but not P2Y11 (260bp). The controls, without reverse transcriptase (noRTs), did not produce any detectable bands.

Optimizing ATP treatment time

In previous studies, ATP treatment was done for 3 h[9], but we wanted to see if a shorter time would suffice for measuring significant ROS production. So we treated cells with ATP for 5 min, 15 min, 30 min, 1 h, and 3 hr. We found that there was a statistically significant increase in ROS production for all conditions when compared to the control, except for the 5 min condition (Fig 2). ATP treatment at 5 min resulted in the greatest amount of ROS production followed by a 15 min treatment, 3 h, 1 h, and finally 30 min. While the 3 h condition was the most statistically significant, and while the 15 min and 30 min conditions were also reasonably good times to use, since our positive control treatment was set at 1 h incubation, we chose to do the same for ATP.

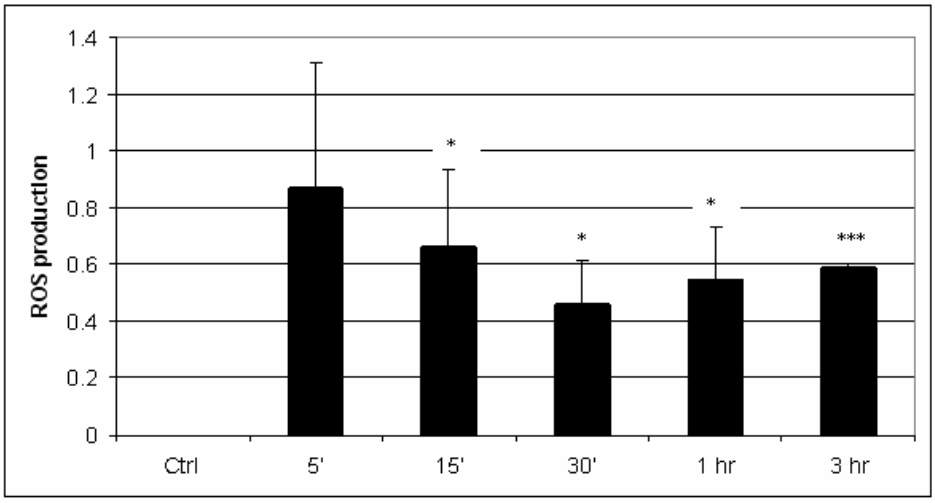

Fig. 2 Optimizing ATP treatment time. There was significant ROS production measured at all time points compared to the control except for 5 min.

ROS production is dependent on ATP ligation of the P2X7 receptor

Previous studies have shown that ATP stimulates the production of ROS. As ATP is a ligand to the P2X7 receptor, we wanted to see if the ROS production is a product of ATP stimulation of the P2X7 receptor[9]. To check this, we used a P2X7-specific antagonist (oxATP) treatment for 30 min before treating the cells with ATP for 1h to see

if there were any effects on ROS production. We also checked to see if inhibition of pannexin-1, a channel-former related to the functioning of the P2X7 receptor and its ligation by ATP, had any effects on ROS production. To do this, we used a peptide mimicker probenecid, which has been shown to block the effects of pannexin-1[10], to treat the cells 10 min before treatment with ATP.

Compared to nigericin (Nig - a K^+ ionophore and known ROS stimulator) [11], the positive control, we found that ATP stimulates similar to greater levels of ROS production. The cells pretreated with oxATP showed an extremely significant decrease in production of ROS, when compared to ATP treatment alone, to a level below that of the control. Also, the cells pretreated with probenecid showed a significant decrease in ROS production to levels similar to that of the control (Fig 3). Both oxATP and probenecid pretreatments to ATP, when compared to oxATP alone, probenecid alone controls, as well as probenecid and oxATP pretreatments to nigericin controls, showed significant ROS production (data not shown). These results suggest that there is dependency on the P2X7 receptor for ATP induced

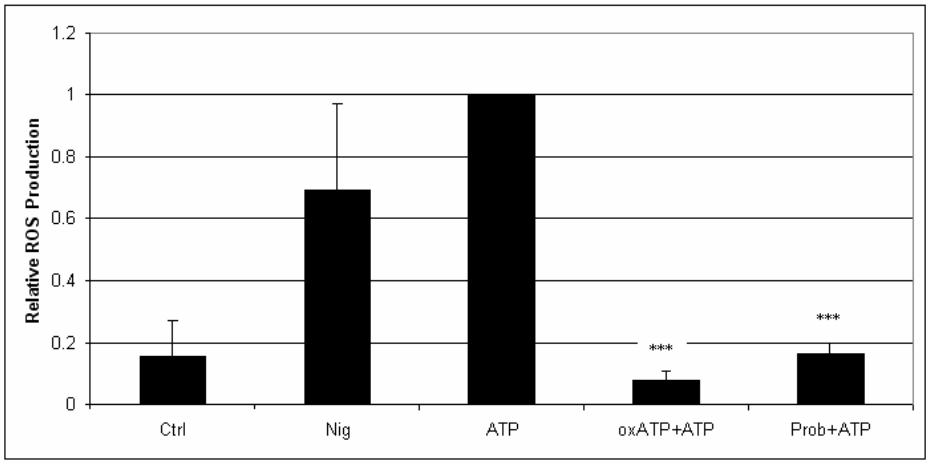

Fig. 3 ROS production in GECs is P2X7 dependent. ATP stimulates greater levels of ROS production than nigericin (positive control). Cells pretreated with oxATP (30 min) and probenecid (10 min) followed by ATP treatment showed an extremely significant decreased production of ROS when compared to ATP treatment alone.

ROS production, and it further suggests that there are effects on the P2X7 pathway when the pannexin channel is blocked.

It has been previously shown that a small concentration of ATP stimulates the P2X4 receptor, whereas high concentrations stimulate the P2X7 receptor[12], so we wanted to confirm that the ROS production is a product of P2X7 stimulation. Also, given the similarity of the nucleotides' structures between ATP, ADP, AMP, and ADO, as well as the fact that the GECs express receptors for all but AMP, we wanted to check if the ROS production due to P2X7 stimulation is purely ATP dependent. Our preliminary results showed that none of the nucleotides besides ATP stimulated ROS production (Fig 4), and therefore the ROS production was indeed due to the ligation of ATP to the P2X7 receptor. However, there was some unexpected ROS production in the ADO condition, though statistically speaking it was 'not quite' significant. Adenosine has been suggested to having effects on immune response due to excessive inflammation, but there hasn't been much research done looking in to the connection between extracellular adenosine and ROS production.

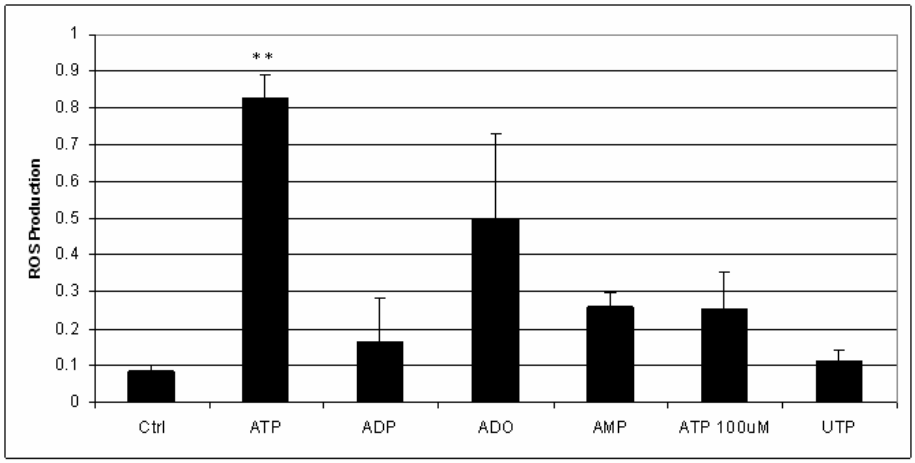

Fig. 4 ROS production is dependent on ATP ligation of the P2X7 receptor. Only ATP treatment stimulated ROS production 'very significantly'. ADO stimulated 'not quite significant' levels of ROS production. The rest of the conditions didn't stimulate significant amounts of ROS production.

Caspase-1 activation in GECs by ATP stimulation is P2X7 dependent

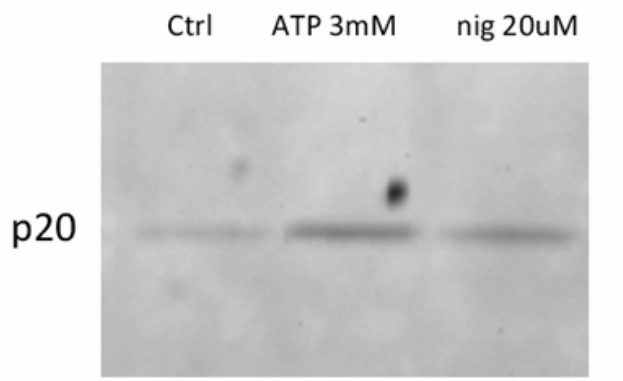

Fig. 5 ATP stimulation causes Caspase-1 activation.

It has been shown previously that the caspase-1 activation in GECs is a result of ATP stimulation[6]. We wanted to see if this activation of caspase-1 is P2X7 dependent. To check this, we pretreated cells with oxATP and probenecid separately, for the same times as stated previously, and again, nigericin was used as the positive control. So far our results are inconclusive as we don't have consistent data replications. We do have data showing that GECs do have activated caspase-1 in the cellular extract. The activated caspase-1 (the p20 band), was found in both ATP and nigericin treatment lanes and with greater intensity than that of the control (Fig 5).

DISCUSSION

In accordance to previous findings, we found that GECs express the P2X7 receptor as well as the pannexin-1 receptor mRNAs. GECs also appear to express other common purinergic receptors, though not P2Y11. The band in the P2Y11 lane didn't correspond to the expected size. The fact that the band that did appear in that lane was around 50bp when the P2Y11 band was expected to appear at 260bp seems to suggest that the primers didn't find any P2Y11 mRNA to bind. Instead the primers more likely dimerized, thus forming the visible band. An alternate rationale for the band in the P2Y11 lane could be attributed to an alternate form or splice of the mRNA, but this idea is can't hold as there have not been any other forms of P2Y11 acknowledged.

Measuring the amount of ROS production in the GECs turned out to be a rather troublesome and required a different method of measuring compared to cervical epithelial cells, HeLas. Flow cytometry was used for measuring ROS production in HeLa cells quite successfully in that there were no artifacts obscuring the results. On the other hand, using flow cytometry to measure the ROS production in GECs did not produce usable results. The fluorescence emitted by the cells, due to an excess of spontaneous ROS production, was so great in the control that the fluorescence emitted by the cells with different treatments could not be compared against them. There were no consistent differences between the negative controls (ctrl, oxATP+ATP, prob+ATP) and the positive controls (nig, ATP): the control seemed to show greater ROS levels than the nigericin and ATP, while the oxATP and probenecid pretreatments also showed high levels of ROS, thus making the results unreliable. We attribute this spontaneous ROS production to the cellular stress caused in the flow cytometry preparation process. The

GECs are tightly adherent cells, so they are probably greatly stressed during the trypsinizing to collect the cells. Also, unlike macrophages, GECs are not suspension cells, and are normally closely adjacent to more GECs, meaning there is a certain amount of cell-to-cell signaling in the normal state. So when they are put in suspension for the flow cell, this in itself could potentially initiate stress responses in the form of ROS production. For this reason we found an alternative method for measuring ROS production in the form of fluorescent microscopy. This method greatly reduced the amount of stress experienced by the cells. However, a different kind of problem emerged for this process. We noted that if there were GECs touching one another on the tissue culture plate (from which we took our pictures); they tended to fluoresce excessively when stimulated by the laser. Only when the cells were spread apart did they act as expected. This, admittedly, doesn't give us much insight into how these cells would act in physiological conditions as cells in the gingiva, however these cells are also not primary cells, which could explain this artifact.

Even though we already knew that a 3 h ATP treatment was enough to cause ROS production, ROS production is transient, hence there could be other treatment times just as effective[9]. The 3 h treatment resulted in an extremely significant amount of ROS production compared to the control although it didn't show the most ROS production, the 5 min treatment did. However, the statistical analysis showed that it wasn't a significant difference, most likely due to the fact that there was such variance across trials. The prep time taken before the fluorescent images can be captured could change the amount of transiently produced ROS in the cell. And, while the 30 min condition showed significant ROS production, under the microscope, there was a less dramatic showing of ROS

production. The choice for the optimal ATP treatment time thus came down to 15 min and 1 h. The 15 min showed greater variance of ROS production across trials than the 1 h, and since our nigericin treatment was already set at 1 h, it seemed our best optimal time would be 1 h.

In order to verify that the ROS production via ATP stimulation was truly P2X7 dependent, the P2X7 antagonist, oxATP was used pre-ATP treatment. There have been no studies showing that oxATP binds to any of the other purinergic receptors. Also, an oxATP treatment before treatment with nigericin, which stimulates ROS production via K+ efflux, didn't yield any significant difference in ROS production when compared to the control. Thus, we can more safely say that oxATP does indeed block the affects of ATP in a P2X7 dependent manner. On top of the ROS production being P2X7 dependent, there are also the effects of the pannexin-1 channel to consider. The pannexin-1 receptor having been found to physically associate with the P2X7 receptor [13] would put forward the possibility of one receptor being vital in the functionality of the other. Probenecid, while being associated with dye-uptake, has been shown to inhibit the pannexin channels and more specifically pannexin-1, while showing no effect on the connexin channels, suggesting that probenecid is at least pannexin-specific[10]. In the same study, it was shown that probenecid attenuated the release of ATP into the extracellular medium. It has been found that the pannexin-1 channels are highly permeable to ATP, and that probenecid stimulates this release of ATP[10]. Unfortunately, there isn't enough research describing the relationship between the pannexin-1 channel and P2X7 that would allow us to explain the affects of the pannexin-1 channel blocking on ROS production.

Even though GECs express the purinergic receptors for the nucleotides except AMP[7], the fact that none of those nucleotides stimulated ROS production should in itself be indicative of a P2X7 dependent ROS formation. The P2Y receptors can be bound by ATP, ADP, and UTP, while the P2X receptors are bound by primarily by ATP. P2X7 is specifically matched to ATP, also, according to our data thus far, only ATP managed to stimulate ROS production. Adenosine seemed to have stimulated a significant amount of ROS production, but statistically speaking, it was 'not quite' significant. There are four adenosine receptors expressed in GECs (data not shown), and they may have a role in the regulation of immune function [14], however, there isn't research showing a connection between the adenosine receptors and ROS production. Further investigation into the effects of adenosine and its receptors on ROS production is necessary.

Confirming caspase-1 activation in GECs via ATP stimulation of the P2X7 receptor has yet to be completed. The most notable issue so far for this part of the project would be the fact that detecting small proteins can be difficult. Not transferring enough protein from gel to membrane or blowing the protein past the membrane can be problematic. At this point, more replications of this experiment is necessary before any conclusions are drawn.

The next step for this project would be to use siRNA to knock down the P2X7 and pannexin-1 receptors to check ROS production and caspase-1 activation under the same conditions as has been presented in this thesis. Then continuing on that stream, we would include *P. gingivalis* and *P. gingivalisΔNDK* infection as additional conditions

concurrently with the conditions we have already investigated. This would give us a fuller understanding of the innate immune response in GECs.

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