The effects of *Pseudomonas aeruginosa* lipopolysaccharides on axonal outgrowth in *Gallus gallus* sympathetic neurons

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Introduction

\textit{Pseudomonas aeruginosa} is a gram negative bacterium that acts as a dangerously antibiotic resistant and adaptive pathogen. It is mainly a threat to immunodeficient patients, especially those with cystic fibrosis, and is one of the leading causes of nosocomial infection (Moradali, Ghods, & Rehm, 2017). Over 80\% of cystic fibrosis patients eventually die from respiratory failure brought on by chronic bacterial infection; especially from \textit{P. aeruginosa} because of its many adaptive mechanisms that enable it to thrive as a biofilm in the mucus build up in the lungs of patients with cystic fibrosis (Bhagirath et. al., 2016). It can be very difficult to treat \textit{P. aeruginosa} infection because treatment is further complicated by the pathogen’s tendency to infect those that are immunodeficient (Hancock & Speert, 2000). \textit{P. aeruginosa} has many virulence mechanisms (Moradali, Ghods, & Rehm, 2017), however, this research will focus on the cellular effects of lipopolysaccharides from the outer membrane of \textit{P. aeruginosa}. \textit{P. aeruginosa} has an outer membrane similar to other gram negative bacteria, comprised largely of lipopolysaccharides (LPS) - molecules that play a critical role in infection (Hancock & Speert, 2000). There are many mechanisms by which LPS can affect mammalian cells, but the most significant is by initiating a pro-inflammatory response in the infected host (Pier, 2007). Gram negative bacteria shear off LPS from their outer membrane continuously. Upon removal from the membrane, LPS gets degraded into O-antigen and lipid A (Jaffer, Wade, & Gourley, 2010). Lipid A activates toll like receptor 4 (TLR4), a g-coupled protein receptor which activates cytokines (Jaffer, Wade, & Gourley, 2010). This activation causes the cell to release large amounts of cytokines, namely tumor necrosis factor alpha (TNFα), that lead to an inflammatory response in living organisms (Jaffer, Wade, & Gourley, 2010).

The inflammatory mechanism triggered by LPS can be quite detrimental to a cell’s survival. Kuhn (2014) studied the effects of pro-inflammatory cytokines in vitro by placing TNFα on polystyrene beads and observing effects when growth cones encountered the polystyrene beads. Growth cones that encountered the TNFα coated beads were paralyzed and collapsed in response, but growth cones that encountered beads without TNFα did not show the same behavior (Kuhn 2014). This study suggests that cytokines, namely TNFα, can inhibit neural growth without causing apoptosis in vitro.
The notion that TNFα leads to cellular stress is supported by investigation into the effects of TLR4, the receptor that moderates the activity of TNFα (Paola et al., 2012). In vitro, Paola et al. used LPS to stimulate TLR4, producing a pro-inflammatory (agonistic) response. This result further supports that TLR4 receptor agonists like LPS lead to cellular stress, presumably by cytokines like TNFα.

It is understood that LPS from gram negative bacteria have the ability to activate the TLR4 receptor, leading to the release of TNFα (Jaffer, Wade, & Gourley, 2010). And it has been shown that TNFα can inhibit the motility and structure of growth cones in vitro (Kuhn 2014). There is yet to be an investigation, however, into whether or not axonal growth will be inhibited by LPS treatment. The aim of this study was to use neurons and glia from dorsal root ganglia (DRGs), harvested from 10 day Gallus gallus embryos, to study the effects of P. aeruginosa LPS on axonal growth. It has been confirmed that DRG neurons and glia express the TLR4 protein (Barajon et al., 2009), so therefore it is reasonable to expect that LPS will bind with TLR4 and elicit the release of TNFα. In light of the proven inhibitory effects of TNFα on growth cone motility and structure, this research tested the hypothesis that LPS would inhibit axonal growth in developing DRG neurons.

Materials and Methods

Tissue Dissection and Cell Plating

Cells from 10 day Gallus gallus embryos were plated on coverslips. Prior to cell plating, coverslips were sterilized with ethanol and dried with Kimwipes. Tissue dissections were performed by Dr. Robert Morris in accordance with the protocols outlined by Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION, a procedure based on the work of Peter J Hollenbeck and modified by Dr Robert Morris (Morris, 2015). Coverslips were treated with laminin and poly-lysine before cell plating and placed in 2 ml F+ growth medium in 35mm petri dishes. F+ growth medium was made by Dr Robert Morris and consisted of L-15, glutamine, glucose, pen/strep, fetal bovine serum (FBS), and nerve growth factor (NGF) (Morris, 2018). After plating, cells were incubated at 37 degrees Celsius for 24 hours before experimental and control conditions were applied.

Cell Treatment and Control Conditions

A 1mg/ml LPS stock solution was prepared by adding 13.3ml of Hanks Balanced Salt Solution (HBSS) to 13.3mg of LPS. In order to create the working solution of 1 microgram/ml, 2 microliters of the stock solution were added to 2ml of F+ medium using a P20 micropipette. To place the solution on the growing cells, a complete medium exchange was performed. All growth medium was removed from the cells using a sterile Pasteur pipette and replaced with the LPS working solution. In order to ensure that any effect was not caused by the medium exchange, a full medium exchange was completed for control conditions. The full medium exchange consisted of removing all growth medium from control plates and replacing it with new growth medium of the same exact composition.

P. aeruginosa LPS solutions were made with F+ growth medium, as LPS will readily dissolve in growth medium (Lipopolysaccharides from Pseudomonas aeruginosa). A concentration of 1 microgram of LPS per ml of growth medium was used with a 30 minute
incubation period, chosen based on the protocols outlined in previous studies (Acosta & Davies, 2008). After 30 minutes of LPS exposure, cells were imaged and put back into incubation at 37 degrees Celsius, and imaged again after 6 hours of further exposure. The addition of the 6 hour treatment was chosen in an effort to determine whether or not longer LPS incubation times have a heightened effect on growing neurons.

**Image Acquisition**

Images were acquired with a Nikon Eclipse T5100 inverted microscope, including a 0.5x adapter mount and a SPOT Idea camera, using SPOT 4.6 software on a Macintosh computer. Images were taken with transmitted light at the 20x objective (20x was chosen over 40x because it allowed for more growth cones to be measured at once than 40x did). Data points were gathered for 1-2 regions consisting of at least 2 growth cones per condition. Growth was measured via time-lapse for 30 minutes, taking an image every 5 minutes without changing the composition of the image.

**Image Analysis**

Images were analyzed with ImageJ version 1.52a, an image analysis software program developed by the National Institute of Health. Images taken during time-lapse photography were imported and organized into image stacks. Growth cones were defined by regions at the end of axons with gray values between 10 and 65. All regions of interest that fit this definition were analyzed. Using this definition, the polygon tool was used to trace each growth cone, and using analyze>measure on the ImageJ toolbar, the growth cone’s center of mass (centroid) was calculated as (X,Y) pixel coordinates. This procedure was repeated for 4 growth cones under each condition, measured every 5 minutes. As the axon grew, the growth cone’s centroid would advance, allowing the axon’s growth to be tracked in pixels. Once these data were acquired, the X value of the initial position was subtracted from the X value of the final position; and the same was done for the initial Y value and the final Y value. This resulted in the total distance that the growth cone traveled across the X axis, as well as the Y axis. Using these two values in the Pythagorean Theorem \(a^2+b^2=c^2\), the hypotenuse, or actual distance traveled by the growth cone in 30 minutes, was calculated. In order to translate these data from pixels to micrometers, an image was taken of a scale bar with 10 micrometer intervals at 20x magnification (the objective used during data collection). In ImageJ, the line tool was drawn from the right side of one interval to the right side of the next interval, a span of 10 micrometers, and measured in pixels. This value became the conversion factor for pixels to micrometers.

**Results**

For the 30 minute control group, 6 growth cones from 3 neurons were measured. For the 6 hour control group, 7 different growth cones from 4 neurons were measured. For the 30 minute LPS-treated group, 8 growth cones from 6 neurons were measured. For the 6 hour LPS-treated group, 9 growth cones from 2 neurons were measured. For each group, 7 images were taken of the same region over the course of 30 minutes. This allowed for the observation of axonal outgrowth over time. By tracking the movement of growth cones’ centroids, micrometers of growth were determined for growing axons.
The phase-contrast light microscopy images (figures 1-2) are indicative of control and experimental conditions, both of which were performed on relatively low density cell cultures. As seen in figure 3, axons showed greater average outgrowth under control conditions than they did for experimental (LPS-treated) conditions. In addition, axons showed greater outgrowth after 6 hours than they did after 30 minutes for both control and experimental conditions. As seen in Figure 4, neurons under control conditions expressed fewer paralyzed growth cones than neurons treated with LPS.

SPSS, a statistical analysis software, was used to complete a factorial ANOVA for both independent variables (LPS vs Control; 30min vs 6hr). The factorial ANOVA did not yield significance for groups, time, nor groups*time. This lack of significance for any relationship implies that the control group did not grow significantly more than the LPS-treated group. In addition, neither 6 hour group (LPS-treated or control) grew significantly more than either 30 minute group. Another factorial ANOVA was computed in order to determine if any of the groups had significantly more paralyzed growth cones than the others.

Figure 1. Cells 30 minutes after full growth medium exchange, representing control conditions. For control conditions, data were gathered from all points within the images that fit the definition for a growth cone mentioned in the section ‘Image Analysis.’ Axons showed more average total growth under control conditions than under experimental conditions. Scale bar (top left) indicates 100 micrometers. Images presented were adjusted in brightness for clarity and consistency in this report, but were not adjusted before data collection.
Figure 2. Cells after 6 hours of LPS exposure representing experimental data conditions. For experimental conditions, data were gathered from all points within the images that fit the definition of a growth cone mentioned in the section ‘Image Analysis.’ Axons showed less average total growth under experimental conditions than under control conditions. Scale bar (top right) indicates 100 micrometers. Images presented were adjusted in brightness for clarity and consistency in this report, but were not adjusted before data collection.
Figure 3. Total average distance traveled by growth cones. Note that the average total growth of the control group was higher than that of the experimental group after both 30 minutes and 6 hours of treatment. Also note that control and LPS-treated groups both exhibited more growth after 6 hours of treatment than they did after 30 minutes.

Figure 4. Number of paralyzed growth cones per group. Note that the control groups had fewer unmoving growth cones than did the LPS-treated group. Groups measured after 6 hours of treatment also displayed more unmoving growth cones than did groups measured after 30 minutes of treatment.
Discussion & Conclusions

This research set forth to test the hypothesis that the presence of \( P. \) \( \text{aeruginosa} \) LPS would inhibit the growth of axons in dorsal root ganglia neurons from 10 day \( Gallus \) \( \text{gallus} \) embryos. As shown by **figure 4**, neurons treated with LPS showed lower average growth than cells not treated with LPS. As shown in **figure 5**, cultures treated with LPS contained more growth cones that exhibited no growth. Neither of these differences in growth were statistically significant and thus do not support the hypothesis. The failure to yield significance, in this case, is most likely attributable to the small sample size of each group.

If these data were significant, however, it would be reasonable to conclude that \( P. \) \( \text{aeruginosa} \) inhibits the axonal outgrowth of \( Gallus \) \( \text{gallus} \) peripheral neurons. If LPS did inhibit axonal outgrowth, it would likely be relatable to the findings that TNFα leads to the collapse of growth cones (Kuhn, 2014). This conclusion would be justifiable based on previous studies that conclude that LPS can activate the TLR-4 receptor and that the TLR-4 receptor regulates the release of TNFα (Jaffer, Wade, & Gourley, 2010). This ability of LPS is well documented, as are many of the manifestations of cellular stress brought on by the release of TNFα (Kuhn, 2014). Without statistical significance or a larger sample size, however, this research is unable to conclude that LPS led to the inhibition of axonal growth.

In the future, a much larger sample size would be needed to conclude that LPS has a significant effect on axonal growth. This study could have also been refined in terms of the quality of microscopy time-lapse images. Some data sets were unusable due to lack of resolution, improper focus, and/or accidental change in the image composition during time-lapse. More consistent and sharper images throughout time-lapse would provide more quantifiable data to reinforce these results. Regardless, these data suggest that LPS from \( P. \) \( \text{aeruginosa} \) may inhibit axonal growth in \( Gallus \) \( \text{gallus} \) peripheral neurons.

The preliminary findings of this study are particularly relevant to the body of research on cystic fibrosis pathology. 80% of cystic fibrosis patients eventually become chronically infected with \( P. \) \( \text{aeruginosa} \) by adulthood, a condition that is often times fatal (Buchanan et al., 2009). The literature surrounding the pathophysiology of cystic fibrosis in the lungs is extensive, however, much less extensive is the literature surrounding the pathophysiology of cystic fibrosis in the nervous system. Patients with cystic fibrosis experience neurological complications at a rate much higher than in the general population, some of which include severe headache, stroke, and seizures (Goldstein et al., 2000). Cystic fibrosis patients have been shown to be more at risk for mental health problems, specifically depression and anxiety (Talwalkar et al., 2017). In addition, cystic fibrosis patients report ADHD symptoms at a rate that is much higher than average (Cohen-Cymerknoh et al., 2018). The neurological complications involved in the experience of cystic fibrosis are not fully understood at this time.

The current study has found preliminary evidence to suggest that \( P. \) \( \text{aeruginosa} \) LPS can inhibit neuronal growth. It has previously been shown that LPS can cross the blood brain barrier (Banks & Robinson, 2010) and that the TLR-4 receptor (critical to the detection of and response to LPS) is prevalent in the nervous system (Barajon et al., 2009). The prevalence of LPS infection in cystic fibrosis patients and the ability of LPS to access the nervous system may imply that LPS could potentially play a role in the neurological dysfunctions associated with cystic fibrosis. Being that this study found preliminary, but not significant, evidence that LPS can inhibit axonal growth, further research into the effects of LPS on neuronal development is
warranted, as it may shed light on some of the complications associated with the treatment of cystic fibrosis.

References Cited


Morris, R. L. (2018). Problem Set 1 (Growth Medium Ingredients)