INTRODUCTION

Acute otitis media is one of the most common infectious diseases, affecting up to 11% of the worldwide population, reflecting between 300 and 700 million patients per year.1 About half the patients affected are children,1 and otitis media (including its chronic forms) is responsible for up to 3.200 deaths per year. 2 About 140 million patients retain a hearing impairment after acute otitis media each year, which can be caused by persisting perforation of the tympanic membrane or suppurative labyrinthitis and subsequent sensorineural hearing loss. 3

ABSTRACT

Objective: To evaluate the effect of Lipopolysaccharide (LPS), a bacterial endotoxin on cochlear microcirculation, and its mode of action.

Methods: Twenty-five Dunkin-Hartley guinea pigs were divided into five groups of five animals each. After surgical preparation, cochlear microcirculation was quantified by in vivo fluorescence microscopy. Placebo or LPS (1 mg, 10 µg, and 100 ng) was applied topically, and microcirculation was measured before and twice after application. A fifth group was pretreated with etanercept, a tumor necrosis factor (TNF) antagonist, and afterward the lowest LPS concentrations that yielded significant results (10 µg) were applied.

Results: In the groups that had been treated with 1 mg and 10 µg LPS, a significant drop in cochlear microcirculation was observed after 30 (.791 ± .089 Arbitrary Units (AU), compared to baseline, and .888 ± .071AU) and 60 (.756 ± .101 AU and .817 ± .124 AU, respectively) minutes. The groups that had been treated with 100 ng LPS and that had been pretreated with etanercept showed no significant change in cochlear blood flow compared to placebo.

Conclusion: Lipopolysaccharide shows a dose-dependent effect on cochlear microcirculation; this effect can already be observed after 30 min. Pretreatment with etanercept can abrogate this effect, indicating that TNF mediates the effect of LPS on cochlear microcirculation.

KEYWORDS

cochlear blood flow, lipopolysaccharide, microcirculation, otitis media, tumor necrosis factor
Additionally, people who suffer chronic hearing loss from otitis media are more likely to suffer from depression or anxiety-related issues.4

Otitis media is commonly caused by a variety of bacteria, among the most common are the gram-positive bacteria Streptococcus pyogenes or pneumoniae, Staphylococcus spp., and the gram-negative bacteria Klebsiella spp., Pseudomonas spp., Proteus spp., Haemophilus influenzae, Moraxella catarrhalis, and Escherichia coli.5,6 Several of these express lipopolysaccharide (LPS), an endotoxin which is situated in the outer membrane of gram-negative bacteria, and which is a considerable inflammation mediator. It has been established that injection of lipopolysaccharide into the middle ear is a viable animal model for acute bacterial otitis media.7,8

When it comes to permanent sensorineural hearing loss, it is commonly believed that this is caused by transition of toxins though the round8 and oval10,11 window membrane, causing inflammation of the cochlea (suppurative labyrinthitis).12 These findings had been reproduced in animal models of otitis media with suppurative labyrinthitis.12

Cochlear microcirculation is an important functional parameter of the cochlea— and impairment of cochlear microcirculation coincides with decreases in partial oxygen pressure14 and increases in hearing thresholds.15,16

There has been evidence that permanent decreases in cochlear microcirculation form the common final pathophysiological pathway of numerous inner ear pathologies, including sudden sensorineural hearing loss,16 endolymphatic hydrops17,18, and noise trauma.15 Until recently, the pathologies of the inner ear were strictly divided into those that were of an inflammatory origin and those that were of an inflammatory origin. However, it has been observed that inflammatory cytokines like tumor necrosis factor (TNF) may impair cochlear blood flow, rendering this division of entities to be inaccurate.19

Hence, we decided to investigate the effect of LPS on cochlear microcirculation and its mode of action.

2 | MATERIALS AND METHODS

All experiments here were done according to state as well as animal protection law and had been registered with the responsible authorities in Göttingen, Lower Saxony (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit LAVES, Oldenburg, Germany; license no. 33.11.42502–04/16/2308), and Munich, Free State of Bavaria, Germany (Regierung von Oberbayern, Munich, Germany; license no. ROB-55.2-2532.Vet_02-17-231).

2.1 | Animals

Animals were healthy female Dunkin-Hartley guinea pigs purchased from Envigo Laboratories, Venray, The Netherlands, aged 8–12 weeks, with a bodyweight of 250–400 g. After an acclimation period of 1 week, animals were included in the experiment.

2.2 | Anesthesia and surgical preparation

The animal model used has been described20 and used extensively. Anesthesia was induced by intramuscular injection of 85 mg/kg bodyweight (b.w.) of ketamine and 8.5 mg/kg b.w. of xylazine. Injections were repeated every 30 min with half of the dosage to keep up anesthesia. Once depth of anesthesia was sufficiently deep, as was controlled by corneal and toe-pinching reflexes, the hair above the ear and the neck was mechanically and chemically removed. After this, about 0.5 ml of lidocaine with epinephrine was injected into the tissue and an i.v. catheter was inserted into the jugular vein. Then, the ear and the muscle tissue covering the temporal bone were removed. The lateral temporal bone was then removed as well as the ossicles and thus the cochlea exposed. Vessels covering the cochlea were removed by simply wiping them off with a microspore, and a rectangular window of approximately 400 × 400 µm was then carefully carved into the cochlea, at the height of the second turn. Then, fluoresceinisothiocyanate-dextrane (FITC, Molecular weight of 500,000, purchased from Sigma-Aldrich, Deisenhofen, Germany) was injected intravenously as a contrast material. Thus, the blood flow of the stria vascularis could be visualized.

2.3 | Microscopy and calculation of cochlear blood flow

Cochlear microcirculation was then recorded using a Leica M205FA binocular microscope with a Leica EL 6000 light source and proprietary Leica Application Suite (all Leica, Wetzlar, Germany) software. (Video S1 Figure 1A,B) The obtained videos were then stored digitally for later off-line quantification of capillary diameter using CapImage (Dr. Zeintl Engineering, Heidelberg, Germany)—a software that has been designed specifically for this purpose. (Figure 1C) Breathing excursions are corrected for manually.

Initially, three representative vessels were chosen at random. In these vessels, at each point in time, capillary diameter and intravascular blood velocity were quantified by measuring each value thrice and then taking the average of the three measurements. Cochlear blood flow was then calculated by the formula that was specifically proposed by Wayland24 for this purpose: \( q = \frac{v}{(v/1.6) \times (d/2)^2 \times \pi} \), where \( v \) is the velocity of the blood flow (in µm/s), \( d \) is the diameter of the representative capillary (in µm), and \( q \) is the blood flow (in µl/s). To correct for interindividual differences, the changes for each animal are reported as relative changes in blood flow, given as arbitrary units (AU).

2.4 | Experimental protocol

Overall, 25 guinea pigs were included in this study. Each one was randomly assigned into one of five groups of five each. After the surgical preparation had been finished, basal values were recorded.
for about 3 min before treatment started. Afterward, the first treatment was then applied topically for 20 min. To achieve this, the bulla was filled with the dissolved LPS (or sterile saline 0.9% as carrier, respectively) until the window carved into the cochlea was covered with a layer of fluid. First treatment consisted either of Placebo (sterile saline 0.9%) or LPS (1 mg, 10 µg, or 100 ng per ml dissolved in sterile saline 0.9%) in various concentrations for 20 min. Then, the bulla was rinsed with saline for approximately 10 min and cochlear microcirculation was then quantified. Following this, the same concentration of LPS was then again applied for another 20 min. Finally, the bulla was rinsed again for 10 min and microcirculation was quantified again. Following this, the animals were euthanized.

In a second part of the experiment, the lowest concentration that yielded a significant decrease in cochlear microcirculation after 30 min of exposure was chosen. Subsequently, the stria vascularis was topically pretreated with etanercept, a TNF-antagonist, as has been reported previously, for 20 min in the same manner as LPS was applied and cochlear microcirculation was quantified. Following this, the lowest concentration of LPS that yielded significant results after 20 min exposure (10 µg/ml) was applied for 20 min and cochlear microcirculation was quantified again.

2.5 | Statistics

To detect significant differences, we fitted linear mixed models that included a random effect for the animal and were estimated using a restricted maximum likelihood approach. A p value <0.05 was considered to be significant. The software used for this was Project R (Build 3.2.5 for Windows, The R Project for Statistical Computing, http://www.r-project.org/).

3 | RESULTS

3.1 | Effect of Placebo on cochlear microcirculation

Topical application of sterile saline leads to no significant changes in cochlear microcirculation, which remained at .998 arbitrary units (AU) ± .060 AU. Subsequent application of placebo again showed no change in cochlear microcirculation, which was at .996 ± .074 AU. (Figure 2/Figure 3, left column).
3.2 | Effect of 1 mg/ml LPS on cochlear microcirculation

Application of 1 mg/ml LPS on cochlear microcirculation leads to a drop in cochlear microcirculation compared to placebo, dropping to .791 ± .089 AU. The following application of the same dosage showed another drop in microcirculation to .756 ± .101 AU. The changes in cochlear blood flow differed significantly compared to placebo. (p < 0.01, Figure 2/Figure 3, columns 2nd to left).

3.3 | Effect of 10 µg/ml LPS on cochlear microcirculation

Application of 10 µg/ml LPS on cochlear microcirculation leads to a drop in cochlear microcirculation to .888 ± .071 AU. After subsequent application, microcirculation dropped further to .817 ± .124 AU. These values were significantly different from those observed in the placebo group. (p < .05, Figure 2/Figure 3, middle columns).

3.4 | Effect of 100 ng/ml LPS on cochlear microcirculation

Application of 100 ng/ml LPS caused cochlear microcirculation to drop to .968 ± .148 AU and subsequently to .893 ± .163 AU after repeated application of LPS. These changes were not significantly different from those observed in the group that was treated with placebo. (Figure 2/Figure 3, 2nd columns from right).

3.5 | Effect of Etanercept / LPS on cochlear microcirculation

Application of etanercept (5 ng/ml) caused cochlear microcirculation to drop .963 ± .155 AU and subsequently after application of 10 µg LPS to .931 ± .147 AU. These changes were not significantly different compared to the group that had been treated with placebo. (Figure 2/Figure 3, columns on the right).

The original dataset including the measures of capillary diameter and intravascular blood velocity is available as online supplemental material to this manuscript.

4 | DISCUSSION/CONCLUSION

Firstly, we have been able to show that exposure of the stria vascularis to lipopolysaccharide is capable of decreasing cochlear blood flow. It has been reported that LPS is capable of decreasing microcirculation in numerous tissues like kidney, liver, gastrointestinal organs, the central nervous system, and muscles. However, this effect has to this day not been demonstrated in the stria vascularis of the cochlea. The terminal vessels of the stria vascularis are pivotal in the upkeep of cochlear function, and impairment of cochlear blood flow leads to a rapid decrease of partial oxygen pressure and subsequent threshold shift and, if persistent, to permanent cellular damage by oxidative stress. Consequently, it is likely that impairment of cochlear blood flow may contribute to the permanent cochlear damage seen in suppurative labyrinthitis.

In addition to this, we were able to show that the effect of LPS on cochlear microcirculation shows dose-dependent effect. Independent of how high the concentration of LPS was applied, we never observed a decrease greater than 20% compared to basal values. Yet this effect was seen faster in those guinea pigs treated with higher concentrations of LPS. This suggests that the effect of LPS on microcirculation is mediated by a cellular receptor that is activated in a stronger fashion when high concentrations of LPS are present. Fittingly, we have also been able to prove that the effects of LPS on cochlear microcirculation can be abrogated by previous application of etanercept. Hence, it seems highly probable that the effects of LPS on cochlear microcirculation are—at least partially—mediated by tumor necrosis factor. TNF is regularly released as a response to stress by fibrocytes of the spiral ligament, as has also been described for fibrocytes in other tissues. TNF has been shown to cause cochlear pericytes to contract and thus reduce cochlear blood flow. Additionally, LPS may directly affect cochlear pericytes, as has been observed in different tissues as well as in cochlear pericytes after TNF exposure in vitro.

It has also been shown that in the central nervous system, pericytes are among the first cells to sustain damage in persistent hypoxia and—if hypoxia persists long enough—may enter a rigor mortis like state, thus persistently impairing capillary blood flow. Considering the similar physiological properties, a similar mechanism seems well probable. In return, a persistent decrease in cochlear...
microcirculation will again cause a persistent decrease in partial oxygen pressure, as both parameters are closely related. Persistent decreases in partial oxygen pressure cause formation of reactive oxygen species, which in turn cause activation of TNF pathways, creating a vicious cycle (Figure 4). This view is further supported by the fact that TNF may cause cochlear inflammation in concentrations that are not sufficient to directly cause apoptosis of hair cells, yet is still able to cause shifts in hearing thresholds. While we are aware that the concentrations that we applied in these experiments were very high—LPS is usually present in significantly lower concentrations—we were able to observe a significant effect on cochlear microcirculation after the rather short application period of 20 min. The longer LPS the was applied, the greater the observed effect became. These observations are very much in line with the proposed theory of a vicious cycle: The higher the initial concentration of LPS, the faster the vicious cycle is activated.

While this is certainly not the only way otitis media may cause cochlear damage in suppurative labyrinthitis, it seems probable that persisting impairment of cochlear microcirculation plays a major role. This view is further supported by the fact that impairment of cochlear microcirculation has also been suggested to be causative of sudden sensorineural hearing loss and acoustic trauma. Assuming that the presence of aforementioned vicious cycle causes an initially reversible threshold shift (impaired microcirculation with decreased partial oxygen pressure and impaired cochlear function), subsequent persistence may cause permanent cochlear damage by inducing apoptosis of inner (and outer) hair cells through reactive oxygen species and TNF. Hence, etanercept is a viable candidate for clinical treatment of otitis media with suppurative labyrinthitis. Consequently, agents that are able to abrogate this vicious cycle may also prove useful in these cochlear pathologies.

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CONFLICT OF INTEREST
The authors Ihler, Freytag, Kloos, Spiegel, Haubner, Canis, Weiss, and Bertlich declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
MB and FI designed the experiment. MC, BGW, and FI secured funding of the experiment. MB, FI, and BK conducted the experiments. SF and MB analyzed the data. MB, JLS, BGW, FH, and MC interpreted the data. MB wrote the manuscript. JLS, BGW, SF, FI, and FH provided critical proofreading and substantial improvement of the manuscript.

DATA AVAILABILITY STATEMENT
Original data are available as online supplementary material.

ORCID
Mattis Bertlich https://orcid.org/0000-0002-6479-5899

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.
Author/s:
Ihler, F; Freytag, S; Kloos, B; Spiegel, JL; Haubner, F; Canis, M; Weiss, BG; Bertlich, M

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