HEARING LOSS

Endotoxemia-mediated inflammation potentiates aminoglycoside-induced ototoxicity

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The ototoxic aminoglycoside antibiotics are essential to treat severe bacterial infections, particularly in neonatal intensive care units. Using a bacterial lipopolysaccharide (LPS) experimental model of sepsis, we tested whether LPS-mediated inflammation potentiates cochlear uptake of aminoglycosides and permanent hearing loss in mice. Using confocal microscopy and enzyme-linked immunosorbent assays, we found that low-dose LPS (endotoxemia) greatly increased cochlear concentrations of aminoglycosides and resulted in vasodilation of cochlear capillaries without inducing paracellular flux across the blood-labyrinth barrier (BLB) or elevating serum concentrations of the drug. Additionally, endotoxemia increased expression of both serum and cochlear inflammatory markers. These LPS-induced changes, classically mediated by Toll-like receptor 4 (TLR4), were attenuated in TLR4-hyporesponsive mice. Multiday dosing with aminoglycosides during chronic endotoxemia induced greater hearing threshold shifts and sensory cell loss compared to mice without endotoxemia. Thus, endotoxemia-mediated inflammation enhanced aminoglycoside trafficking across the BLB and potentiated aminoglycoside-induced ototoxicity. These data indicate that patients with severe infections are at greater risk of aminoglycoside-induced hearing loss than previously recognized.

INTRODUCTION

Severe Gram-negative bacterial infections, including meningitis, bacteraemia, and respiratory infections in cystic fibrosis, are treated with aminoglycoside antibiotics like gentamicin (1–3). These drugs can induce permanent and debilitating hearing loss, particularly in neonates. About 80% of 600,000 admissions into neonatal intensive care units (NICU) in the United States receive aminoglycosides each year (4). The rate of hearing loss in NICU graduates (from all etiologies) is 2 to 4% compared to 0.1 to 0.3% of full-term births from congenital etiologies (5).

Aminoglycoside-induced ototoxicity could contribute substantially to this increased rate of hearing loss in the NICU population. The irreversibility of hearing loss, particularly in pediatric cases before language development, has extensive quality-of-life implications (6–9).

The mechanisms by which circulating aminoglycosides cross the blood-labyrinth barrier (BLB) into the cochlea remain unconfirmed. We previously reported that, in vivo, these drugs predominantly cross the BLB into the stria vascularis and are trafficked via marginal cells located on their apical membranes and induce hair cell death (10–13).

Serious infections induce systemic inflammatory response syndrome, elevating serum concentrations of nitric oxide, vasoactive peptides, and inflammatory proteins that can modulate the vascular permeability of the blood-brain barrier (BBB) (14–16). Vasoactive peptides also modulate cochlear uptake of gentamicin across the BLB (17). However, most studies of ototoxicity involve healthy preclinical models, and the effect of induced systemic inflammation on ototoxicity has only recently been reported (18). Here, we used bacterial lipopolysaccharide (LPS) to induce endotoxemia in a classic experimental model of sepsis and inflammation (19) in mice to test the hypothesis that systemic inflammation modulates cochlear concentrations of aminoglycosides and inflammatory markers, and exacerbates aminoglycoside-induced ototoxicity.

RESULTS

Fluorescently-tagged gentamicin [gentamicin–Texas Red (GTTR)] is an excellent tracer of gentamicin in vivo (10, 13, 17, 20–23). By conjugating Texas Red to gentamicin (450 to 477 daltons, three isoforms), the hydrophobicity of the resulting conjugate (1151 to 1179 daltons) is increased, whereas serum pharmacokinetics are slowed, providing greater spatiotemporal imaging and a higher signal-to-noise ratio in heterogeneous cellular structures like the cochlea compared to radio-labeled aminoglycosides or immunohistochemistry (10, 13, 24–26).

The cytoplasmic intensity of GTTR fluorescence is dose-dependent unlike gentamicin immunofluorescence, where the greater abundance of epitope binding sites can overwhelm the number of available antibodies (13, 20). Thus, changes in cochlear uptake of aminoglycosides can be quantitatively assayed using GTTR and confocal microscopy (13, 17, 20, 23).

Strial uptake of GTTR is enhanced by LPS-induced endotoxemia

In Dulbecco’s phosphate-buffered saline–treated (DPBS)–treated mice that received an intraperitoneal injection of GTTR 1 hour before fixation, intense fluorescence delineated the stria capillaries of the cochlear lateral wall, with moderate diffuse fluorescence distributed throughout the stria vascularis (marginal cells, intrastrial tissues, and basal cells; Fig. 1, A and B), as previously described (13). The spiral ligament fibrocytes exhibited reduced intensities of GTTR fluorescence compared to strial cells (Fig. 1, A and B, and fig. S1A), also as seen previously (13). Mice intravenously injected with LPS (1 mg/kg) 24 hours before an intraperitoneal injection of GTTR 1 hour before fixation had...
more intense GTTR fluorescence in all regions of the cochlear lateral wall compared to DPBS-treated mice (Fig. 1, A and B). DPBS- or LPS-treated mice injected with hydrolyzed Texas Red (hTR) 1 hour before fixation displayed negligible fluorescence within the lateral wall, as previously described (13, 22).

The mean pixel intensity of GTTR fluorescence was obtained from xy optical sections and plotted for specific regions of interest [ROIs; marginal cells, intrastrial tissues (minus capillary structures), basal cells, and spiral ligament fibrocytes] at 1 and 3 hours after GTTR injection (Fig. 1C and fig. S1). In DPBS-treated mice, absolute fluorescence was greatest in marginal cells, with stepwise decreasing fluorescence in intrastrial and basal cells, with a significant drop in spiral ligament fibrocytes (fig. S1A; P < 0.05). Strial and spiral ligament fluorescence was significantly greater in basal segments compared to that in apical segments for each ROI (fig. S1A; P < 0.01).

GTTR fluorescence was dose-dependently elevated in selected cell types with increasing LPS dose (Fig. 1C and fig. S1B). At 1 hour, significant increases in GTTR fluorescence were observed in every ROI of basal lateral wall segments dosed with LPS (1 mg/kg or higher) (Fig. 1C and fig. S1B; P < 0.01 or P < 0.005). A similar pattern of increased uptake was observed in the apical coil but with more variable significance (fig. S1B; P > 0, P < 0.05, P < 0.01, or P < 0.005). At 3 hours, GTTR fluorescence in lateral wall cells continued to show dose-dependent elevations with increasing LPS dose, reaching significance at LPS of 2.5 and 10 mg/kg (Fig. 1C and fig. S1B; P < 0.05 or P < 0.01).

The rank order of GTTR fluorescence intensity in individual cell types was maintained with increasing LPS dose, with more variable degrees of significance with increasing LPS dose and time (fig. S1A). Strial GTTR fluorescence was significantly greater for each ROI in basal segments compared to that in apical segments in DPBS- or LPS-treated mice (fig. S1A; P < 0.01). These data indicate that LPS dose-dependently increased GTTR trafficking across the BLB, particularly in the basal coil of the cochlea, the region most associated with the onset of drug-induced hearing loss and sensory cell death (27, 28).

In cochlear outer hair cells (OHCs), LPS-induced increases in GTTR fluorescence were consistently observed at 1 hour after GTTR injection compared to DPBS-treated mice, particularly at higher LPS doses (>1 mg/kg), and trended toward significance at higher doses of LPS (≥2.5 mg/kg) at 3 hours after GTTR injection (fig. S2, A and B). In apical coils, LPS did not consistently modulate GTTR fluorescence in OHCs 1 hour after GTTR injection and trended toward significance at 3 hours with higher doses of LPS (≥1 mg/kg). These data suggest that LPS accelerated GTTR entry into basal OHCs and that dynamic increases in GTTR uptake kinetics at later time points, or by apical OHCs, were dissipated by the low doses of GTTR used here.

**Fig. 1.** Cochlear lateral wall uptake of GTTR is enhanced by LPS. (A) In xy planes of the cochlear lateral wall 1 hour after GTTR injection, F-actin labeling (green) revealed tight junctions (arrowheads) between marginal cells (MC), with amorphous labeling in basal cells (BC; arrows). In DPBS-treated mice, intense GTTR fluorescence (red) distinguished strial capillaries (c), with less intense fluorescence in marginal cells, intrastrial layer (IS), and basal cells of the stria vascularis. The spiral ligament (SL) fibrocytes presented substantially less intense GTTR fluorescence compared to strial cells. LPS-treated mice displayed more intense GTTR fluorescence in the lateral wall (right panel) compared to DPBS-treated mice (left panel). (B) A focal series of xy planes through marginal cells, intrastrial tissues, basal cells, and fibrocytes at successively lower xy planes in the z axis, 1 hour after GTTR injection. LPS-treated mice exhibited more intense GTTR fluorescence in grayscale (right panels) compared to DPBS-treated mice (left panels). Scale bar, 50 μm. (C) Mean pixel intensities of GTTR fluorescence in lateral wall ROIs (excluding capillary structures) are dose-dependently increased with increasing doses of LPS at 1 and 3 hours after GTTR injection (relative to DPBS-treated mice at 1 hour), with statistical significance in every cell type at 1 hour of LPS (1 mg/kg or higher dose) (*P < 0.05, **P < 0.01, ***P < 0.001, Wilcoxon signed-rank test; error bars, SEM; n as in table S1).
Renal function is impaired at higher doses of LPS
Endotoxemia and sepsis affect vascular function in multiple organ systems and can induce decreased glomerular filtration rates and renal dysfunction (29). Because GTTR is readily taken up by renal proximal tubules (21, 23), decreased glomerular filtration rates and renal dysfunction should reduce proximal tubule uptake of GTTR. As an internal control, we assessed renal GTTR fluorescence intensities during endotoxemia. In DPBS-treated mice, 1 or 3 hours after GTTR injection, GTTR fluorescence was localized as intense puncta close to the lumen and as diffuse fluorescence in the cytoplasm of proximal tubule cells, whereas distal tubule cells had visibly less diffuse fluorescence and no puncta (fig. S3A), as seen previously (21). In LPS-treated mice, diffuse GTTR fluorescence within proximal tubule cells at 1 hour was significantly reduced only at LPS of 10 mg/kg compared to that in DPBS-treated control tissues (fig. S3C; P < 0.001). At 3 hours, cytoplasmic fluorescence in proximal tubule cells of GTTR-treated mice was markedly increased compared to that at 1 hour (fig. S3C) due to longer exposure. However, at 3 hours, only mice treated with LPS (10 mg/kg) had significantly less cytoplasmic GTTR fluorescence compared to DPBS-treated mice (fig. S3; P < 0.05), as expected during endotoxemic shock (29).

Low-dose LPS increases cochlear but not serum concentrations of aminoglycosides
Given that LPS (10 mg/kg) can decrease both glomerular filtration rates (29) and renal uptake of GTTR (fig. S3), we assessed serum concentrations of GTTR 24 hours after LPS administration using immunoturbidimetry. Serum concentrations of GTTR were significantly higher in mice dosed with LPS (2.5 and 10 mg/kg) than in DPBS-treated mice at 1 or 3 hours after GTTR injection (Fig. 2A and tables S1 and S2; P < 0.01 and P < 0.05, respectively). Serum concentrations of GTTR in mice dosed with LPS (0.1 and 1 mg/kg) were not statistically different from controls at 1 or 3 hours of GTTR exposure (Fig. 2A and table S2). Serum concentrations of GTTR in mice dosed with LPS at 2.5 mg/kg were significantly higher than in mice treated with LPS at 0.1 and 1 mg/kg at both 1- and 3-hour time points (Fig. 2A and table S2; P < 0.05 and P < 0.01, respectively). To verify that serum concentrations for unconjugated gentamicin were not altered by exposure to LPS (1 mg/kg) for 24 hours, we used enzyme-linked immunosorbent assays (ELISAs). Both GTTR and gentamicin serum concentrations were unchanged in LPS-treated mice compared to those in DPBS-treated mice at 1- and 3-hour time points after drug injection (Fig. 2, B and D). Serum gentamicin was lower at 3 hours after injection, whereas GTTR has a longer serum half-life, as described previously (13). In contrast, cochlear concentrations of both GTTR and gentamicin were significantly increased in LPS-treated mice compared to those in DPBS-treated mice at both 1 and 3 hours after injection (Fig. 2, C and E; P < 0.05).

Six mice at higher LPS doses died within 24 hours after LPS injection (table S1), as predicted (30). However, LPS at 1 mg/kg was not fatal, as expected (30), yet induced acute weight loss (fig. S4A) associated with endotoxemia and sepsis (31). Given that LPS at 1 mg/kg did not increase serum concentrations, yet elevated cochlear concentrations, of GTTR and gentamicin, we used LPS at 1 mg/kg for subsequent experiments.

Low-dose LPS does not increase paracellular flux across the BLB
Endotoxemia can change the volume of distribution for drugs, including gentamicin (32). hTR (also known as sulforhodamine 101; molecular mass, 679) is a membrane-impermeant fluorophore (33, 34). We used hTR to test whether exposure to LPS for 24 hours enhanced paracellular flux across the BLB into the interstitial spaces of the stria vascularis and spiral ligament (35), using neonatal (P6) mice with an immature BLB as a positive control (21, 36). In P6 mice treated with the hTR for 1 hour, fluorescence was distributed throughout stria tissues, with weaker intensities in stria marginal and basal cells (Fig. 3A and fig. S5A). The spiral ligament of P6 mice exhibited less fluorescence compared to stria tissues (fig. S5A; P < 0.01). In adult DPBS- and LPS-treated mice exposed to hTR for 1 hour, significantly less fluorescence was observed in stria cells and spiral ligament fibrocytes
Twenty-four hours after LPS injection, serum histamine concentrations potentially increased strial vascular permeability to aminoglycosides. Vasoconstrictive serotonin is associated with strial vasodilation and potentially increases histamine and serotonin concentrations of most tested inflammatory proteins were not elevated (or were below the limit of detection) (fig. S7A), consistent with previous findings (42). Although IL-6 and MIP-1α concentrations remained significantly elevated compared to those in DPBS-treated controls, they were substantially lower compared to those in the 6-hour time point (Fig. 4A and fig. S7A). In cochlear homogenates of C57BL/6 mice 24 hours after LPS injection, concentrations of all tested inflammatory proteins were significantly elevated, particularly IL-6, IL-8, and MIP-1α (Fig. 4A). In cochlear homogenates, LPS significantly elevated protein concentrations of all early-phase proinflammatory markers, particularly IL-6 and IL-8, but not IL-1β (Fig. 4B). LPS significantly elevated the anti-inflammatory cytokine IL-10 in serum but not in vascular-perfused cochlear homogenates [Fig. 4, A (P < 0.05) and B, respectively]. Exposure to gentamicin for 3 hours did not modulate serum or cochlear inflammatory protein concentrations (Fig. 4, A and B), indicating that gentamicin (at the doses used here) was not a confounding factor. Overall, LPS-induced changes in the serum concentrations of acute-phase proinflammatory proteins were reflected in cochlear homogenates, except for IL-1β and the anti-inflammatory cytokine IL-10 (Fig. 4, A and B).

Twenty-four hours after LPS injection in C57BL/6 mice, plasma concentrations of most tested inflammatory proteins were not elevated (or were below the limit of detection) (fig. S7A), consistent with previous findings (42). Although IL-6 and MIP-1α concentrations remained significantly elevated compared to those in DPBS-treated controls, they were substantially lower compared to those in the 6-hour time point (Fig. 4A and fig. S7A). In cochlear homogenates of C57BL/6 mice 24 hours after LPS injection, concentrations of all tested inflammatory proteins were significantly elevated, particularly IL-6, IL-8, and MIP-1α (Fig. 4A and fig. S7A). These findings suggested that LPS triggers robust

LPS binds to and activates Toll-like receptor 4 (TLR4) to induce the secretion and transcription of acute-phase inflammatory cytokines and chemokines that orchestrate immune responses (40, 41). Given that LPS enhanced cochlear uptake of aminoglycosides, we assessed whether LPS modulated serum and cochlear expression of acute-phase inflammatory markers. Six hours after injection, LPS significantly elevated serum concentrations of all acute-phase proinflammatory proteins tested: TNFα (tumor necrosis factor α), IL-1α (interleukin-1α), IL-1β, IL-6, IL-8 (also known as KC, CXCL1), MIP-1α (macrophage inflammatory protein–1α), and MIP-2α (Fig. 4A). In cochlear homogenates, LPS significantly elevated protein concentrations of all early-phase proinflammatory markers, particularly IL-6 and IL-8, but not IL-1β (Fig. 4B). LPS significantly elevated the anti-inflammatory cytokine IL-10 in serum but not in vascular-perfused cochlear homogenates [Fig. 4, A (P < 0.05) and B, respectively]. Exposure to gentamicin for 3 hours did not modulate serum or cochlear inflammatory protein concentrations (Fig. 4, A and B), indicating that gentamicin (at the doses used here) was not a confounding factor. Overall, LPS-induced changes in the serum concentrations of acute-phase proinflammatory proteins were reflected in cochlear homogenates, except for IL-1β and the anti-inflammatory cytokine IL-10 (Fig. 4, A and B).

Fig. 3. LPS does not alter BLB permeability but vasodilates basal strial capillaries. (A) The relative mean intensities of hTR fluorescence in marginal cell (MC), intrastrial tissue (IS), basal cell (BC), and spiral ligament (SL) layers from P6 pups were significantly elevated compared to the same ROIs in adult mice. There was no difference in hTR fluorescence of lateral wall ROIs between DPBS- and LPS-treated adult mice (*P < 0.05, ***P < 0.001, two-way analysis of variance (ANOVA) with Bonferroni post hoc tests; n = 6 cochleae per group; error bars, SD). Absolute fluorescence intensities are shown in fig. S5A. (B and C) In P6 mice, the lumen of strial capillaries, revealed by phallloidin labeling, was larger than in adult DPBS-treated mice (endothelial cells indicated by white arrowheads). (D) Twenty-five hours after LPS treatment, a subpopulation of strial capillaries were dilated compared to DPBS-treated mice (C). Scale bar, 20 μm.

(E) Strial capillary diameters in P6 mice were wider than those in DPBS-treated adults (see also Table 1). LPS-treated adult mice had a subset of dilated strial capillaries, resulting in a bimodal distribution. (F) LPS also dilated a subset of strial capillaries in C3H/HeOuJ mice compared to DPBS-treated C3H/HeOuJ mice. (G) In TLR4-hyporesponse C3H/HeJ mice, LPS dilated fewer strial capillaries compared to LPS-treated control C3H/HeOuJ mice (F), resulting in an asymmetrical bimodal distribution. A Gaussian regression curve fit was applied to obtain the bimodal peak means in Table 1.
Means of the bimodal distributions in Fig. 3. A Gaussian regression curve fit was applied to obtain the peak of apical strial capillary diameters in LPS-treated C57BL/6 mice was significantly greater compared to that in DPBS-treated C57BL/6 mice (P < 0.0001). LPS also dilated a subset (50%) of strial capillaries in C3H/HeJ mice. In TLR4-hyporesponsive C3H/HeJ mice, LPS dilated a smaller subset (22%) of strial capillaries. The population of capillary diameters in LPS-treated C3H/HeJ mice was significantly smaller compared to that in LPS-treated C3H/HeOuJ mice (P < 0.0001). The population of capillary diameters in LPS-treated C3H/HeJ mice was significantly greater compared to that in DPBS-treated C3H/HeJ mice (P < 0.0001), one-way ANOVA with Tukey post hoc tests. In apical coils, LPS dilated a subset (45%) of strial capillaries in C57BL/6 mice. The population of apical strial capillaries in LPS-treated C57BL/6 mice was significantly greater compared to that in DPBS-treated C57BL/6 adult mice (P < 0.0001). A Gaussian regression curve fit was applied to obtain the peak means of the bimodal distributions in Fig. 3.

Table 1. Vasodilation of strial capillaries by LPS. In basal coils, strial capillary diameters in P6 pups were significantly larger than those in DPBS-treated C57BL/6 adult mice (**P < 0.001). LPS dilated a subset (45%) of strial capillaries in adult mice. The population of capillary diameters in LPS-treated C57BL/6 mice was significantly greater compared to that in DPBS-treated C57BL/6 mice (P < 0.0001). LPS also dilated a subset (50%) of strial capillaries in C3H/HeOuJ mice. In TLR4-hyporesponsive C3H/HeJ mice, LPS dilated a smaller subset (22%) of strial capillaries. The population of capillary diameters in LPS-treated C3H/HeJ mice was significantly greater compared to that in DPBS-treated C3H/HeJ mice (P < 0.0001). LPS dilated a subset (45%) of strial capillaries in C3H/HeJ mice. In TLR4-hyporesponsive C3H/HeJ mice, LPS dilated a smaller subset (22%) of strial capillaries. The population of capillary diameters in LPS-treated C3H/HeJ mice was significantly smaller compared to that in LPS-treated C3H/HeOuJ mice (P < 0.0001). One-way ANOVA with Tukey post hoc tests. In apical coils, LPS dilated a subset (45%) of strial capillaries in C57BL/6 mice. The population of apical strial capillaries in LPS-treated C57BL/6 mice was significantly greater compared to that in DPBS-treated C57BL/6 adult mice (P < 0.0001). A Gaussian regression curve fit was applied to obtain the peak means of the bimodal distributions in Fig. 3.

Table 2. Effect of LPS on serum concentrations of histamine and serotonin. Serum histamine concentrations were not affected by increasing doses of LPS. Serum serotonin concentrations were significantly decreased at all LPS doses compared to DPBS-treated mice (P < 0.005, **P < 0.001, Mann-Whitney U test; n in table S1 at 3 hours after GTTR injection).

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<tr>
<th>LPS dose (mg/kg)</th>
<th>Histamine (ng/ml ± SEM)</th>
<th>Serotonin (ng/ml ± SEM)</th>
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<tr>
<td>0</td>
<td>33 ± 3</td>
<td>2770 (±100)**</td>
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<tr>
<td>0.1</td>
<td>25 ± 3</td>
<td>2135 (±285)**</td>
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<tr>
<td>1.0</td>
<td>24 ± 4</td>
<td>579 (±157)**</td>
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<tr>
<td>2.5</td>
<td>28 ± 3</td>
<td>830 (±246)**</td>
</tr>
<tr>
<td>10</td>
<td>36 ± 6</td>
<td>837 (±329)**</td>
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Inflammatory responses both systemically and in the cochlea within 6 hours. Furthermore, 24 hours after LPS injection, cochlear continued to exhibit significantly elevated concentrations of tested inflammatory proteins (Fig. 5A), akin to that induced by middle ear administration of LPS or bacteria (43, 44). To determine whether increased cochlear inflammatory protein concentrations were due to local (parenchymal) gene transcription, mRNA levels were assayed 6 or 24 hours after LPS injection (with or without gentamicin). At 6 hours, LPS significantly increased mRNA expression (by fivefold or greater) for all eight inflammatory genes tested, particularly Il-6, Il-8, and Mip-1α (Fig. 4C). Gentamicin alone did not modulate cochlear mRNA expression of any inflammatory genes tested (Fig. 4C).

Twenty-four hours after LPS injection, cochlear mRNA levels remained significantly elevated for most inflammatory genes tested, except Tnfα and Il-1β (Fig. S7G), with greater increases for later-expressing acute-phase cytokines: Il-6, Il-8, and Mip-2α (Fig. 5B and fig. S7G). These data show that LPS challenge up-regulated cochlear mRNA transcription for inflammatory proteins within 6 hours, with sustained mRNA transcription for later-expressing acute-phase inflammatory proteins at 24 hours, as in other organs during endotoxemia including the liver, lung (41), brain (45), and eye (46).

**LPS-mediated inflammation is reduced in TLR4-hyporesponsive C3H/HeJ mice**

The C3H/HeJ mouse strain is homozygous for an inactivating short-nucleotide polymorphism in TLR4, resulting in greatly attenuated inflammatory responses to LPS exposure (47). Twenty-four hours after LPS injection, plasma concentrations of all tested inflammatory proteins in C3H/HeJ mice, and the control C3H/HeOuJ mouse strain, were not different from those in DPBS-treated mice of the same strain (fig. S7, A to C). Unlike control C3H/HeOuJ mice, C3H/HeJ mice did not experience significant weight loss (fig. S4B; P < 0.001).

LPS significantly elevated cochlear homogenate concentrations of all acute-phase proinflammatory proteins 24 hours after injection in control C3H/HeOuJ and C57BL/6 mice compared to those in DPBS-treated mice of the same strains (Fig. 5A and fig. S7, D to F; P < 0.05). Several inflammatory proteins (Tnfα, Il-6, Il-8, MIP-1α, and MIP-2α) were more elevated in C57BL/6 mice compared to those in C3H/HeOuJ mice after LPS treatment (Fig. 5A). Crucially, several, mostly later-expressing acute-phase inflammatory proteins (IL-1α, Il-6, Il-8, and MIP-1α) were significantly attenuated in cochlear homogenates of LPS-treated C3H/HeJ mice compared to those in LPS-treated C3H/HeOuJ and C57BL/6 mice (Fig. 5A; P < 0.05).

Similar trends were seen with cochlear mRNA levels for tested inflammatory genes for both C3H/HeOuJ and C3H/HeJ mice after LPS treatment. LPS significantly increased cochlear mRNA expression of most inflammatory markers tested (not Il-1β) in C3H/HeOuJ mice, particularly later-expressing acute-phase proinflammatory cytokines: Il-6, Il-8, Mip-1α, and Mip-2α (fig. S7H). In contrast, LPS did not modulate cochlear mRNA expression for tested proinflammatory markers in C3H/HeJ mice (fig. S7I). When mRNA expression was compared between strains, there was attenuated mRNA expression for later-expressing acute-phase proinflammatory cytokines (Il-8, Mip-1α, and Mip-2α) in LPS-treated C3H/HeJ cochlea compared to that
in LPS-treated C57/BL6 and C3H/HeOuJ mice, and also for that in II-6 compared to that in LPS-treated C57BL/6 mice (Fig. 5B). mRNA expression of early-phase proinflammatory genes (Tnfa, Il-1α, and Il-1β) was weakly or not significantly elevated in all three mouse strains. mRNA for the anti-inflammatory cytokine Il-10 was transcribed at significantly higher levels in C3H/HeJ mice compared to that in control C3H/HeOuJ or C57BL/6 mice (Fig. 5 and fig. S7, G to I; \( P < 0.05 \)). These data demonstrate that cochlear expression of acute-phase inflammatory markers was up-regulated in LPS-treated control C3H/HeOuJ and C57BL/6 mice, and this up-regulation was attenuated for later-expressing inflammatory markers 24 hours after LPS injection (Fig. 5).

Cochlear lateral wall uptake of GTTR is attenuated in endotoxemic C3H/HeJ mice

Given that LPS-mediated inflammatory responses are attenuated in TLR4-hyporesponsive C3H/HeJ mice, we hypothesized that LPS-enhanced GTTR uptake would also be attenuated in the cochlear lateral wall of TLR4-hyporesponsive C3H/HeJ mice. We injected C3H/HeJ and control C3H/HeOuJ mice with LPS and, 24 hours later, injected GTTR. We found significantly enhanced GTTR fluorescence in strial cells and fibrocytes of LPS-treated C3H/HeOuJ mice compared to that in DPBS-treated C3H/HeOuJ mice (Fig. 6 and fig. S8A; \( P < 0.05 \)). LPS also significantly enhanced GTTR fluorescence in strial cells (but not fibrocytes) in LPS-treated C3H/HeJ mice compared to that in DPBS-treated C3H/HeJ mice (Fig. 6 and fig. S8B; \( P < 0.05 \)). Crucially, however, LPS-enhanced GTTR uptake was significantly attenuated (\( P < 0.05 \)) in marginal cells, intermediate cells, and fibrocytes, with a downward trend in basal cells, in TLR4-hyporesponsive C3H/HeJ mice compared to that in control C3H/HeOuJ mice (Fig. 6). The residual expression of inflammatory markers and cochlear uptake of GTTR in C3H/HeJ mice was likely due to the activation of other innate immune system receptors, such as TLR2 (48). These data indicate that TLR4-mediated inflammation mediated (at least in part) LPS-enhanced cochlear uptake of GTTR.

LPS significantly dilated a subset of strial capillaries in C3H/HeOuJ mice compared to DPBS-treated C3H/HeOuJ mice, as we observed for C57BL/6 mice (Fig. 3, E and F, and Table 1; \( P < 0.0001 \)). Endotoxemia also significantly dilated a smaller subset of strial capillaries in TLR4-hyporesponsive C3H/HeJ mice compared to DPBS-treated C3H/HeJ mice (Fig. 3G and Table 1; \( P < 0.0001 \)). However, the degree of capillary dilation in LPS-treated C3H/HeJ mice was significantly attenuated compared to that in LPS-treated C3H/HeOuJ mice (Fig. 3G and Table 1; \( P < 0.0001 \)).
95% confidence level. See also fig. S6.

mice after one-way ANOVA with Dunnett's test; *, significant difference compared to C3H/HeOuJ and C57BL/6 mice (fig. S11A and table S3), as expected (27). Endotoxemia exacerbated the frequency range of kanamycin-induced threshold shifts (12 to 32 kHz) compared to age-matched control mice not treated with kanamycin (fig. S11A and table S3).

The 3-week recovery time point is a well-established primary benchmark for many preclinical ototoxicity studies (27, 49, 50). At 21 days after treatment, kanamycin-treated mice exhibited a significant permanent threshold shift (PTS) only at 32 kHz compared to DPBS- and LPS-treated mice. Mice that received both LPS and kanamycin had greater and more significant PTS at 16, 24, and 32 kHz compared to kanamycin-treated mice or mice treated with DPBS or LPS alone (Fig. 7A and table S5). Mice receiving both LPS and kanamycin also had significant PTS at 12 kHz compared to mice treated with DPBS or LPS alone and at 8 kHz compared to mice treated with LPS alone (Fig. 7A and table S5). During the 3-week recovery period, kanamycin-induced threshold shifts did not change significantly at individual frequencies between time points (fig. S11, A to C). However, there was a loss of significant threshold shifts at 16 and 24 kHz in kanamycin-treated mice at 10 and 21 days after treatment compared to that in DPBS- and LPS-treated mice that were present 1 day after treatment (fig. S11, A to C). Endotoxemia, however, increased the degree of kanamycin-induced threshold shifts at 21 days and over a wider frequency range (8 to 32 kHz) compared to 1 day after treatment (fig. S11, A to C). This likely reflected the onset of slight, nonsignificant threshold shifts in DPBS- and LPS-treated mice at 10 and 21 days after treatment (fig. S11, A to C). Endotoxemia significantly exacerbated kanamycin-induced PTS.

Drug-induced PTS and sensory hair cell death occur in a dose-dependent fashion (27). To verify whether endotoxemia exacerbated kanamycin-induced hair cell death, we obtained cytococheleograms of cochlear hair cell survival at 21 days after treatment (Fig. 7B and tables S7 and S9). DPBS- and LPS-treated mice had minimal hair cell loss, mostly at the extreme basal (very high frequency) region of the cochlea. Kanamycin induced OHC loss over a wider frequency range in basal cochlear locations, but this was not significant compared to age-matched control mice. LPS-induced endotoxemia significantly enhanced kanamycin-induced OHC loss, and over a much wider frequency range (8 to 64 kHz), compared to non-endotoxemic mice. These data indicate that endotoxemia exacerbated kanamycin-induced OHC death.

We then used hTR to test whether chronic LPS and kanamycin exposure altered the flux of membrane-impermeant hTR across the BLB. Significant threshold shifts were present immediately after chronic treatment with kanamycin, with or without LPS, at 16, 24, and 32 kHz compared to DPBS- and LPS-treated mice (figs. S11D and S12 and table S6). Cytococheleogram data revealed significantly greater OHC loss in the basal (32 to 64 kHz) regions of LPS + kanamycin–treated mice compared to that in all other groups (fig. S11E and table S8). Nonetheless, no differences in hTR fluorescence intensity were detected in strial cells or spiral auditory brainstem response (ABR) threshold shifts. Given that chronic dosing with gentamicin is systemically lethal to mice, we used a related aminoglycoside—kanamycin—with a well-established protocol (27). One group of C57BL/6 mice received LPS (1 mg/kg) the day before kanamycin treatment and on the 5th and 10th day during a 14-day course of kanamycin treatment (700 mg/kg, twice daily). ABR thresholds were obtained from age-matched mice 1, 10, and 21 days after kanamycin treatment, and shifts from pretreatment thresholds were determined (Fig. 7, A and B, figs. S9 to S11, and tables S3 to S5). At all time points, DPBS- and LPS-treated mice had negligible threshold shifts. One day after chronic dosing, kanamycin induced significant threshold shifts at 16, 24, and 32 kHz compared to DPBS- and LPS-treated mice (fig. S11A and table S3), as expected (27). Cytococheleogram data revealed significantly greater OHC loss in the basal (32 to 64 kHz) regions of LPS + kanamycin–treated mice compared to that in all other groups (fig. S11E and table S8). Nonetheless, no differences in hTR fluorescence intensity were detected in strial cells or spiral auditory brainstem response (ABR) threshold shifts. Given that chronic dosing with gentamicin is systemically lethal to mice, we used a related aminoglycoside—kanamycin—with a well-established protocol (27). One group of C57BL/6 mice received LPS (1 mg/kg) the day before kanamycin treatment and on the 5th and 10th day during a 14-day course of kanamycin treatment (700 mg/kg, twice daily). ABR thresholds were obtained from age-matched mice 1, 10, and 21 days after kanamycin treatment, and shifts from pretreatment thresholds were determined (Fig. 7, A and B, figs. S9 to S11, and tables S3 to S5). At all time points, DPBS- and LPS-treated mice had negligible threshold shifts. One day after chronic dosing, kanamycin induced significant threshold shifts at 16, 24, and 32 kHz compared to DPBS- and LPS-treated mice (fig. S11A and table S3), as expected (27). Cytococheleogram data revealed significantly greater OHC loss in the basal (32 to 64 kHz) regions of LPS + kanamycin–treated mice compared to that in all other groups (fig. S11E and table S8). Nonetheless, no differences in hTR fluorescence intensity were detected in strial cells or spiral auditory brainstem response (ABR) threshold shifts. Given that chronic dosing with gentamicin is systemically lethal to mice, we used a related aminoglycoside—kanamycin—with a well-established protocol (27). One group of C57BL/6 mice received LPS (1 mg/kg) the day before...
bars, 95% CI derived from Student's test.

Fig. 6. LPS-induced GTTR uptake by lateral wall cells is attenuated in TLR4-hyporesponsive C3H/HeJ mice. The fold change in GTTR intensity in LPS-treated mice over DPBS-treated mice is shown. GTTR fluorescence was significantly enhanced in strial marginal (MC), intermediate (IC), and basal (BC) cells, as well as fibrocytes (FC) of LPS-treated C3H/HeOuJ mice compared to that in DPBS-treated C3H/HeOuJ mice. LPS also significantly enhanced GTTR fluorescence intensities in strial cells (but not fibrocytes) in LPS-treated C3H/HeJ mice compared to that in DPBS-treated C3H/HeJ mice. Note that LPS-induced GTTR uptake was significantly attenuated (P < 0.05) in marginal cells, intermediate cells, and fibrocytes in C3H/HeJ mice compared to that in C3H/HeOuJ mice (*P < 0.05; n = 8 per bar; error bars, 95% CI derived from Student's t test; significance was determined if 95% CI did not overlap with 1; **P < 0.05, unpaired one-way t test between strains; see fig. S8 for raw data).

Fig. 7. Chronic endotoxemia potentiates kanamycin ototoxicity. (A) Three weeks after chronic LPS (or DPBS) exposure ± kanamycin (see fig. S13), ABR threshold shifts for LPS-only mice (n = 5) were not different from DPBS-treated mice (n = 4). Kanamycin alone (n = 5) induced a small but significant PTS at only 32 kHz (**P < 0.01) compared to DPBS-treated mice. Mice that received LPS + kanamycin (n = 6) had significant PTS at 16, 24 (##P < 0.001), and 32 kHz (##P < 0.005) compared to those treated with kanamycin, DPBS, or LPS only (**P < 0.01). Mice receiving LPS + kanamycin also had significant PTS at 12 kHz compared to those treated with DPBS or LPS only (##P < 0.05 and P < 0.01, respectively), or LPS-only mice at 8 kHz (##P < 0.05). Error bars, SD. All statistical results were produced using two-way ANOVA with Bonferroni post hoc correction with 95% family-wise confidence intervals. (B) Cytocochleogram for mice in (A) revealed that OHC loss in the basal cochlear regions was greater and over a wider frequency range in LPS + kanamycin–treated mice compared to that in mice treated with LPS, DPBS, or kanamycin alone. Mean cochlear length = 6.84 ± 0.79, SD mm. Error bars, 95% CI derived from Student's t test. See also figs. S9 to S11 and tables S5 and S7 for statistical comparisons using two-way ANOVA Bonferroni post hoc correction with 95% family-wise confidence intervals.

Acute LPS-induced endotoxemia does not alter ABR thresholds

Trans tympanic injection of LPS can induce ABR threshold shifts in a dose–dependent manner ([33, 34]). Systemic LPS (0.5 mg/kg per day for 2 days) did not change ABR thresholds or endolymphatic potentials ([18]). Because chronic LPS alone did not induce ABR threshold shifts at 6, 16, or 27 days after the last LPS injection (fig. 7 and figs. S9 to S11), we tested whether acute systemic LPS exposure modulated auditory thresholds. Twenty-four hours after injection with LPS (1 mg/kg), no significant threshold shifts were observed within, or between, DPBS- or LPS-treated mice groups (fig. S13). The persistence of sensitive auditory function during acute LPS challenge suggests that the physical integrity of the BLB remained intact, because physical disruption of the BLB is thought to impair sensitive cochlear performance ([35]).

DISCUSSION

The easy availability and low cost of aminoglycosides contribute to their frequent use worldwide ([28]). Clinical use of aminoglycosides is limited because of the risk of acute nephrotoxicity and, more critically, permanent hearing loss. The risk of otoxicity is proportional to the dose and duration of aminoglycoside therapy ([28]). Additional factors predisposing patients to aminoglycoside-induced ototoxicity include age, renal dysfunction, mitochondrial mutations, and concurrent exposure to other ototoxic drugs (like loop diuretics) or noise ([55–60]).

We used LPS-induced inflammation as a model for aminoglycoside pharmacotherapy of severe Gram-negative bacterial
infections. LPS binds to ubiquitous TLR4 receptors to initiate immune response signaling cascades (40, 41). Lysis of bacteria by aminoglycosides and immune cells releases LPS and other bacterial ligands into the interstitial and vascular fluids, potentiating the inflammatory response (the Jarisch-Herxheimer reaction) (61, 62).

We found that low doses of LPS (≤1 mg/kg) significantly increased the expression of acute-phase inflammatory markers in serum, plasma, and cochlear tissues, mimicking low-grade sepsis. Furthermore, acute or chronic endotoxemia did not modulate the paracellular flux of membrane-impermeant hTR across the BLB, nor attenuate cochlear function. Nonetheless, LPS-induced endotoxemia increased cochlear concentrations of GTTR and gentamicin, without modulating the serum concentrations of these compounds. Simultaneous exposure to chronic endotoxemia and kanamycin significantly increased PTS and OHC death compared to age-matched mice treated with kanamycin, LPS, or DPBS alone.

Endotoxemia and cochlear inflammation

Until recently, the cochlea had been considered an immunologically privileged compartment (63). Here, we show that endotoxemia can elevate cochlear expression of inflammatory markers. Although vascular inflammatory proteins could potentially be trafficked across the BLB, significantly elevated serum concentrations of IL-1β and IL-10 were not reflected in cochlear tissues, implying that the BLB is not passively permeable to serum inflammatory proteins. Endotoxemia also significantly increased cochlear mRNA expression of inflammatory proteins. A similar parenchymal response to endotoxemia has been observed in ocular, pulmonary, and cerebral tissues (42, 45, 46). The cochlear expression of inflammatory markers was greatly attenuated in LPS-treated mice with hyporesponsive TLR4, as was strial vasodilation and GTTR uptake. Thus, endotoxemia-induced inflammation appears to be associated with changes in BLB physiology that enhanced cochlear loading with gentamicin.

Because cochleae are pooled from several mice to determine cochlear inflammatory marker protein concentrations and mRNA levels, it was not possible to correlate any potential tonotopic gradient in inflammatory protein expression with GTTR uptake, auditory threshold shifts, and OHC loss in the basal regions of the cochlea. To accomplish this correlational analysis will require development of more sensitive biochemical or quantitative immunofluorescence techniques.

Endotoxemia alone did not induce ABR threshold shifts, corroborating a recent study showing that low-dose LPS exposure has little effect on endolymphatic potentials and auditory thresholds (18). Disruption of the physical integrity of the strial BLB is thought to elevate ABR thresholds (35). This implies that the BLB remained physically intact, even though endotoxemia enhanced aminoglycoside trafficking across the BLB.

 Trafficking across the BLB

Several mechanisms have been proposed for the trafficking of small compounds like aminoglycosides across tight junction–coupled endothelial barrier layers, like the BBB and the BLB (see fig. S14). Paracellular flux across the BLB is not thought to occur under normal physiological conditions (10, 64) but may arise during inflammation, as has been reported for the BBB (15). Pathophysiological opening of paracellular routes can occur by immune cell–dependent and immune cell–independent mechanisms (65). In our panel, IL-8 and MIP-2α, strong chemotactic signals for immune cell recruitment and diapedesis into the parenchyma beyond the blood vessels (66, 67), were greatly elevated. Immune cells are capable of secreting cytotoxic molecules that disrupt tight junctions between adjacent endothelial cells, opening paracellular trafficking routes (68). Alternatively, the tight junction coupling between adjacent endothelial cells could break down independently of immune cell activity, allowing paracellular flux through disrupted endothelial tight junctions (69). Although we did not use markers for immune cell–mediated injury or electron-dense tracers, the lack of hTR flux into the intrastriatal tissues of adult mice contraindicated a major contribution by paracellular trafficking during acute endotoxemia. Furthermore, chronic endotoxemia with or without chronic kanamycin treatment did not increase hTR flux across the adult strial BLB, corroborating analogous experiments with mannitol after chronic ototoxic drug treatment (64).

Unlike membrane-impermeant hTR, GTTR rapidly traversed the adult BLB into strial tissues and entered cochlear hair cells within 30 min (10, 13, 21), suggestive of transcellular trafficking across the BLB. This could occur via several mechanisms characterized in other cell systems. Aminoglycosides and GTTR can permeate through nonelective cation channels, including the NET channel expressed by hair cells (11, 12) and TRPV4 channels expressed by endothelial cells (70, 71). The sodium-glucose transporter 2 traffics aminoglycosides into cells and facilitates aminoglycoside–induced cytotoxicity (72). LPS treatment can up-regulate endothelial cation channel expression (73). If endotoxemia increases the expression of aminoglycoside-permeant ion channels or transporters, it will be crucial to determine whether these channels enable LPS-enhanced trafficking of aminoglycosides across the BLB.

Endocytotic and transcytotic trafficking across the BLB has been described previously (74) and is increased during endotoxemia in noncochlear capillary beds (75). Although aminoglycosides strongly interact with negatively charged phospholipid membranes (76), hydrophobic passage (or diffusion) through the membrane is slow (77). How endotoxemia potentiates aminoglycoside trafficking routes across the BLB remains to be determined.

Endotoxemia exacerbates ototoxicity

Chronic kanamycin dosing induced PTS at only 32 kHz. Endotoxemia significantly exacerbated the degree of kanamycin-induced PTS at 32 kHz and at additional lower frequencies. Furthermore, endotoxemia significantly potentiated the degree of kanamycin-induced OHC death, predominantly in the basal region of the cochlea. These data support previous observations that bacteremia and hyperthermia (an experimental model for sepsis–induced fever) enhanced aminoglycoside–induced ototoxicity in humans and mice, respectively (78, 79). Endotoxemia also potentiated aminoglycoside–induced nephrotoxicity (80) and heightened the degree of cisplatin–induced PTS (81).

Kanamycin–induced OHC loss occurred in a narrower tonotopic range than drug–induced PTS, as reported previously (27, 82). This mismatch between the broader frequency ranges of PTS and narrower tonotopic regions of OHC loss has been attributed to functional dysregulation of the stria vascularis, hair cell mechanotransduction, and/or synaptic activity in surviving hair cells (12, 82, 83). Although partial recovery of auditory function after kanamycin treatment has been described previously, it only occurred in regions with lower threshold shifts and greater OHC survival (82). The basis for this partial recovery is thought to be drug clearance from cochlear tissues, facilitating the repair of hair cell and strial physiology incurred during sublethal toxicity to resume optimal auditory function (25, 82, 84). However, once threshold shifts exceed >40 dB (akin to the LPS + kanamycin group), no
functional or anatomical recovery of hair cells occurred, resulting in a PTS (82, 85).

Endogenous inflammatory responses to Gram-negative bacterial infections are crucial to controlling infection and host survival. However, the clinical use of the life-saving bactericidal aminoglycosides can inadvertently heighten the existing inflammatory response via bacteriolytic release of endotoxins, that is, the Jarisch-Herxheimer reaction (61, 62), to potentiate both cochlear uptake of aminoglycosides and ototoxicity. Thus, efforts to ameliorate aminoglycoside-induced ototoxicity (and nephrotoxicity) using pharmacotherapeutic agents, for example, D-methionine (86), should also aim to demonstrate otoprotection in preclinical models with induced inflammation.

The progression of acute-phase inflammation is characterized by changing expression patterns of specific inflammatory proteins over time (40, 41), whereas the onset of aminoglycoside-induced ototoxicity in mice requires several days of administration (27). We tested inflammation-enhanced cochlear uptake of aminoglycosides only at 24 hours, and this potentiated uptake could be greater at other time points after induction of endotoxemia. We induced endotoxemia using LPS from Escherichia coli, yet LPS from other bacteria can also activate TLR4. It will be important to determine whether other TLR signaling cascades (particularly TLR3 for viremia) also potentiate aminoglycoside-induced ototoxicity. If inner ear inflammation increases the penetration of non-ototoxic antibiotics (for example, cephalosporins) into the cochlea, then this phenomenon could better preserve auditory and vestibular function during bacterial labyrinthitis (87).

We conclude that endotoxemia-induced inflammation potentiated ototoxicity by increasing aminoglycoside trafficking across the BLB into the cochlea. In toto, these data suggest that patients receiving aminoglycoside pharmacotherapy for life-threatening bacterial infections are at greater risk of ototoxicity than previously recognized.

MATERIALS AND METHODS

Study design

The objective was to test the hypothesis that LPS-induced inflammation increased cochlear concentrations of aminoglycosides without renal dysfunction or increased serum aminoglycoside concentrations in C57BL/6 mice. We verified LPS-induced systemic and cochlear inflammation using multiplex ELISAs and quantitative reverse transcription polymerase chain reaction (RT-PCR) (44) in C57BL/6 mice and in mice with hypofunctional TLR4 activity (C3H/HeJ) and their strain control (C3H/HeOuJ). We then tested whether LPS-induced inflammation exacerbated aminoglycoside-induced ototoxicity using a well-established protocol for C57BL/6 mice (27). All experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University (OHSU) and followed the ARRIVE (Animals in Research: Reporting In Vivo Experiments) reporting guidelines (88).

Serum and cochlear concentrations of GTTR, gentamicin, serotonin, and histamine

GTTR was prepared as before (20). Mice, chosen at random, received a tail vein injection of DPBS or LPS, followed by an intraperitoneal injection of GTTR (2 μg/g), gentamicin (20 μg/g), or hTR (2 μg/g molar equivalent to GTTR) 24 hours later (fig. S15). LPS-treated mice received one dose (0.1, 1, 2.5, or 10 mg/kg) of LPS (E. coli serotype 0111:B4). One or 3 hours after GTTR, gentamicin, or hTR injection, cardiac blood samples were obtained before cardiac perfusion with DPBS followed by 4% paraformaldehyde, and cochlea and kidneys were immersion-fixed. Fixed tissues were counter-labeled with Alexa 488–conjugated phalloidin and examined by confocal microscopy for fluorophore intensity or capillary diameter analyses by operators blinded to treatment groups (20). Serum concentrations of the gentamicin epitope of GTTR were obtained by OHSU Diagnostic Services (13). Serum and cochlear concentrations of gentamicin or GTTR were also assayed by ELISA according to the manufacturer’s instructions (EuroProxima) to determine concentrations. Serum serotonin and histamine concentrations were obtained using ELISA kits (Rocky Mountain Diagnostics).

Inflammatory protein and mRNA analyses at 6 hours

C57BL/6 mice were randomly grouped and treated as described in fig. S16. For multiplex ELISAs, cochleae were homogenized before performing multiplex ELISAs in duplicate (44). For quantitative RT-PCR, excised cochleae were placed in RNAlater (Ambion) and stored at 80°C.

Tissue RNA was extracted, reverse-transcribed using an RT² first-strand kit, prepared for RT-PCR using custom PCR arrays optimized for reaction conditions, primers, and probes (SABIsciences), and analyzed using the SABiosciences PCR Array Data Analysis Web Portal (44).

Inflammatory protein and mRNA analyses at 24 hours

Twenty-four mice for each strain (C57BL/6, C3H/HeOuJ, and C3H/HeJ) were randomly grouped and treated as shown in fig. S16. For ELISAs, six cochleae from three mice per sample were pooled in protein extraction buffer before multiplex ELISA, in triplicate. Absolute protein concentrations from DPBS-treated mice were subtracted from LPS-treated mice, and the 95% CIs derived from Student’s t test were propagated. For mRNA levels, RNAs from each pair of cochleae were obtained and complementary DNA samples analyzed in triplicate using RT² qPCR Primer Assays read on an Applied Biosystems Step One Plus qRT-PCR. Relative expression levels were calculated using the ΔΔCt method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Otototoxicity studies

ABRs to pure tones were used to obtain measures of cochlear function before and after treatments to determine threshold shifts (20, 72). For toxicity studies, mice were randomly divided into four groups: (i) DPBS only, (ii) LPS only, (iii) kanamycin only, and (iv) LPS plus kanamycin (fig. S17). Mice received kanamycin (700 mg/kg) (or DPBS) twice daily for 14 consecutive days (27). Mice received a tail vein injection of LPS (1 mg/kg) (or DPBS) the day before kanamycin treatment and on the 5th and 10th day during kanamycin treatment. ABRs were obtained before kanamycin treatment and 1, 10, and 21 days after kanamycin treatment. ABRs were obtained before kanamycin treatment and 1, 10, and 21 days after kanamycin treatment before fixation and cytocochleogram analyses by operators blinded to treatment groups (27). Additional mice in each group received hTR for 1 hour after ABR testing on day 14 of chronic LPS and/or kanamycin treatment, before fixation and processing for hTR fluorescence intensity and cytocochleogram analyses as described above. Neonatal pups (P6) were used as positive controls.

Statistical analyses

Statistical analyses were chosen on the basis of data under analysis. In brief, Wilcoxon signed-rank test was used for GTTR intensity analyses, the Mann-Whitney U test for ordinal variables (for example, Fig. 2A), and Student’s unpaired t test for single-variable analyses (for example,
Fig. 2, B to E). For vasodilation analyses, we used one-way ANOVA with Tukey post hoc tests. For ELISA studies in C57BL/6 mice, we used a one-way ANOVA with Bonferroni multiple comparison correction with family-wise 95% confidence levels, whereas comparisons of mRNA levels in C57BL/6 mice were determined as significant if the 95% CI did not overlap with 1 (control-treated baseline). To compare ELISA and mRNA data between strains, a one-way ANOVA with either Bonferroni or Dunnett’s post hoc tests and a family-wise 95% confidence level was used. For ABR and cytocochleogram analyses, we used two-way ANOVA with Bonferroni or Tukey post hoc tests. The statistical analyses used, number of replicate measurements, and number of mice are stated in each figure legend or in the Supplementary Materials. P values <0.05 were considered significant.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Number of mice in each condition for Figs. 1 and 2A and fig. S1.


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Endotoxemia-mediated inflammation potentiates aminoglycoside-induced ototoxicity

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Editor's Summary

**Inflammation potentiates antibiotic-induced hearing loss**

Aminoglycoside antibiotics are essential for treating life-threatening infections, despite the known risk of permanent hearing loss by damaging cochlear sensory cells in the inner ear. Because severe infections also trigger widespread inflammation, Koo and colleagues tested the effect of inflammation on aminoglycoside-induced hearing loss as a model of clinical dosing with these drugs. Systemic inflammation increased cochlear uptake of aminoglycosides and, surprisingly, cochlear expression of inflammatory markers in mice. Inflammation also greatly exacerbated aminoglycoside-induced hearing loss and cochlear sensory cell death in sick mice compared to healthy mice. These findings may help clinicians to choose the appropriate pharmacotherapy for patients with severe infections to better protect lifelong hearing health, and encourage the development of otoprotective drugs that also demonstrate efficacy against aminoglycoside-induced ototoxicity during infection-induced inflammation.

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