Tumor Necrosis Factor-α Enhances Microvascular Tone and Reduces Blood Flow in the Cochlea via Enhanced Sphingosine-1-Phosphate Signaling

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Background and Purpose—We sought to demonstrate that tumor necrosis factor (TNF-α), via sphingosine-1-phosphate signaling, has the potential to alter cochlear blood flow and thus, cause ischemic hearing loss.

Methods—We performed intravital fluorescence microscopy to measure blood flow and capillary diameter in anesthetized guinea pigs. To measure capillary diameter ex vivo, capillary beds from the gerbil spiral ligament were isolated from the cochlear lateral wall and maintained in an organ bath. Isolated gerbil spiral modiolar arteries, maintained and transfected in organ culture, were used to measure calcium sensitivity (calcium-tone relationship). In a clinical study, a total of 12 adult patients presenting with typical symptoms of sudden hearing loss who were not responsive or only partially responsive to prednisolone treatment were identified and selected for etanercept treatment. Etanercept (25 mg s.c.) was self-administered twice a week for 12 weeks.

Results—TNF-α induced a proconstrictive state throughout the cochlear microvasculature, which reduced capillary diameter and cochlear blood flow in vivo. In vitro isolated preparations of the spiral modiolar artery and spiral ligament capillaries confirmed these observations. Antagonizing sphingosine-1-phosphate receptor 2 subtype signaling (by 1 µmol/L JTE013) attenuated the effects of TNF-α in all models. TNF-α activated sphingosine kinase 1 (Sk1) and induced its translocation to the smooth muscle cell membrane. Expression of a dominant-negative Sk1 mutant (Sk1S225P) eliminated both baseline spiral modiolar artery calcium sensitivity and TNF-α effects, whereas a nonphosphorylatable Sk1 mutant (Sk1S225A) blocked the effects of TNF-α only. A small group of etanercept-treated, hearing loss patients recovered according to a 1-phase exponential decay (half-life=1.56±0.20 weeks), which matched the kinetics predicted for a vascular origin.

Conclusions—TNF-α indeed reduces cochlear blood flow via activation of vascular sphingosine-1-phosphate signaling. This integrates hearing loss into the family of ischemic microvascular pathologies, with implications for risk stratification, diagnosis, and treatment. (Stroke. 2010;41:2618-2624.)

Key Words: signal transduction ■ transfection ■ etanercept ■ sphingosine kinase 1 ■ cochlear microcirculation
The fine spatial distribution of blood flow is controlled by the cochlear microvascular networks, which ultimately feed the stria vascularis. This architecture creates a direct relationship between the location of flow restriction and the extent of auditory symptoms (that is, range of frequencies affected). Thus, more proximal flow restrictions are prone to yield widespread auditory symptoms (pantonal hearing loss), whereas the symptoms resulting from distal flow restriction would be more discrete (hearing loss in a narrow frequency range). Despite the vital function of cochlear blood flow, remarkably little is known about the molecular mechanisms that regulate cochlear microvascular tone or its potential connection to hearing loss. We have previously described that tone and resistance to blood flow in the SMA are principally controlled by the constrictive phospholipid, sphingosine-1-phosphate (SIP). The SIP-generating enzyme sphingosine kinase 1 (Sk1) is activated by TNF-α, which, in principle, imbues this cytokine with the potential to profoundly disturb the cochlear microcirculation.

The present study proposes that inflammation, via TNF-α-dependent activation of SIP signaling, can reduce cochlear blood flow. To advance this vascular hypothesis, we assessed the effect of TNF-α/SIP signaling on cochlear blood flow/arteriolar tone (intravital microscopy of the cochlear microvascular) and capillary diameter (ex vivo spiral ligament preparation). To provide evidence for a vascularly based mechanism in humans, the recovery profiles of a selected, small group of etanercept-treated hearing loss patients were compared with those of kinetics predicted for a primary vascular origin.

**Methods**

**Animal Models**

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All experimental protocols complied with Canadian, American, and German federal animal protection laws and were approved by the respective institutional animal care and use committees.

Intravital fluorescence microscopy to measure blood flow and capillary diameter in the stria vascularis was accomplished via intravenous injection of fluorescein isothiocyanate–labeled dextran into anesthetized guinea pigs in combination with standard intravital fluorescence microscopy techniques. To measure capillary diameter ex vivo, capillary beds from the gerbil spiral ligament were isolated from the cochlear lateral wall and maintained in an organ bath. Because direct measurement of capillary diameter yields a poor signal-to-noise ratio, an indirect method based on the measurement of red blood cell (RBC) movement was employed with the potential to profoundly disturb the cochlear microcirculation.

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**Assessment of Auditory Function in Etanercept-Treated Patients**

The use of human subjects in this study was approved by the human studies ethics committee of the Technische Universität München, Munich, Germany (No. 2172/08). A detailed description of the selection/exclusion criteria, presurgery assessments, and a patient composite is located in the online-only Data Supplement. A total of 12 adult patients presenting with the typical symptoms of sudden hearing loss who were unresponsive or only partially responsive to prednisolone treatment were identified and selected for etanercept treatment. Etanercept (25 mg s.c.) was self-administered twice a week for 12 weeks.

Hearing function was assessed by pure-tone audiometry, speech reception threshold, and word recognition. A pure-tone-average investigation was performed at 9 frequencies between 250 and 6000 Hz. Hearing improvement was defined as an improvement of (1) hearing from baseline, (2) ≥20 dB in the pure-tone air-conduction thresholds in at least 3 of the tested frequencies, and (3) ≥15 dB at 2 consecutive frequencies.

**Statistics**

Data represent mean±SEM for n experiments. Experimental groups were compared by paired or unpaired Student’s t tests, or ANOVA followed by a t test with a Bonferroni correction for multiple comparisons, as appropriate. Differences were considered significant at P<0.05.

**Results**

TNF-α superfusion (1 ng/mL, 20 minutes) rapidly reduced guinea pig cochlear blood flow (determined as RBC velocity) from 132.9±4.0 to 86.8±6.0 μm/s, with a half-maximal effect reached after 7 minutes (Figure 1B). SIP2 receptor blockade (10 μmol/L JTE013; 10-minute superfusion before addition of TNF-α) fully prevented this response (Figure 1B). The TNF-α–stimulated reduction in cochlear blood flow was associated with a decrease in capillary diameter (from 9.0±0.4 to 6.7±0.2 μm), which was also prevented by SIP2 receptor blockade (Figure 1C). Systemic effects of the treatments can be excluded, because arterial pressure remained constant throughout the experiment (data not shown).

Consistent with Figure 1C, TNF-α constricted gerbil spiral ligament capillaries ex vivo (length=260±23 μm; diameter=7.2±0.2 μm) in an SIP2 receptor–dependent manner (that is, inhibition by 10 μmol/L JTE013; Figure 2B). Accordingly, SIP also stimulated capillary constriction (Figure 2C and supplemental Figure I) with an estimated median effective concentration of 4.7 μmol/L (supplemental Figure II).

In the isolated gerbil SMA, a model with the unique advantage in that it can be transfected, TNF-α (1 ng/mL, 2 hours) significantly increased vasoconstriction in response to calcium under depolarizing conditions (plateau of maximal tone; Tmax con=26±1; TNF-α=41±4% of maximal diameter, n=5; Figure 3A). TNF-α had no effect on resting SMA tone. Etenancept (a competitive TNF-α inhibitor) cotreatment prevented the TNF-α–mediated increase in SMA tone (Tmax con=21±1; TNF-α+etanercept=19±1% of maximal diameter, n=5; Figure 3B). JTE013 (1 μmol/L, 30 minutes) also fully reversed the effect of TNF-α (Tmax con=37±5, TNF-α=50±5, TNF-α+JTE013=33±4% of maximal diameter; Figure 4A); however, unlike etanercept (Figure 3B), JTE013 significantly decreased basal calcium sensitivity (Tmax con=23±3, JTE013=11±2% of maximal diameter;
The 1 μmol/L JTE013 concentration appeared to specifically inhibit S1P responses (online-only supplemental results). In SMAs expressing GFP-tagged Sk1 (GFP-Sk1) within their smooth muscle cells, TNF-α (1 ng/mL, 2 hours) stimulated
translocation of GFP-Sk1 from a primarily cytosolic location (Figure 5A) to the plasma membrane (Figure 5B). This translocation serves as a marker of Sk1 activation.22,23

Expression of a dominant-negative Sk1 mutant (Sk1G82D) in SMA smooth muscle cells, which would be expected to reduce endogenous S1P levels, virtually abolished calcium sensitivity (diamax = 89 ± 5 μm, n = 5 paired observations). B, Coincubation with etanercept (Etc, 1 μg/mL), a TNF-α inhibitor, prevented the TNF-α-stimulated increase in SMA calcium sensitivity (diamax = 91 ± 7 μm, n = 5 paired observations). *Denotes a significant difference (P < 0.05) in the dose-response relations.

Figure 4. The S1P2 receptor subtype is critical for the TNF-α-mediated calcium sensitivity increase in the SMA. A, Treatment of SMAs with 1 μmol/L JTE013 (30 minutes) after TNF-α stimulation (1 ng/mL, 2 hours), which increased calcium sensitivity, reversed the effect of TNF-α (maximal diameter [diamax] = 91 ± 4 μm, n = 9 paired observations). B, In the absence of TNF-α, JTE013 inhibitor treatment resulted in reduced calcium sensitivity (ie, a rightward shift in the calcium/tone relation), compared with control SMAs (diamax = 97 ± 9 μm, n = 5 paired observations). *Denotes a significant difference (P < 0.05) in the dose-response relations.

(Sk1G82D) prevented the TNF-α–dependent increase in calcium sensitivity (Figure 4F; Tmax of Sk1G82D = 25 ± 3, Tmax of Sk1G82D + TNF-α = 23 ± 2%; n = 5). Unlike SMAs expressing Sk1G82D, SMAs expressing Sk1G82S retained a significant level of calcium sensitivity (compare Figure 5C and 5F). In HEK293T cells, TNF-α signals via TNF-α receptor–associated factor 2 to activate Sk1.25 In the SMA, however, expression of an Sk1 mutant lacking its TNF-α receptor–associated factor 2 binding motif (TB2-Sk1) failed to prevent the TNF-α–dependent increase in calcium sensitivity (Tmax of TB2-Sk1 = 22 ± 5, Tmax of TB2-Sk1 + TNF-α = 29 ± 4; n = 6, P < 0.05).

To translate our rodent data into a human background, we recruited a small number of otherwise unresponsive sudden-hearing-loss patients for off-label treatment with etanercept (a TNF-α inhibitor). Patients were subcategorized into “acute” (symptoms present <3 months) and “chronic” (symptoms present for >3 months) hearing loss cases. Etanercept treatment improved 6 of the 7 acute hearing loss cases but only 2 of the 7 chronic hearing loss cases. The kinetics data for acute hearing loss patients who responded to treatment were perfectly fit by a 1-phase, exponential decay curve (calculated half-life = 1.56 ± 0.20 weeks, r² = 0.9916; Figure 6); after 12 weeks of treatment improved 6 of the 7 acute hearing loss cases but only 2 of the 7 chronic hearing loss cases. The kinetics data for acute hearing loss patients who responded to treatment were perfectly fit by a 1-phase, exponential decay curve (calculated half-life = 1.56 ± 0.20 weeks, r² = 0.9916; Figure 6); after 12 weeks of
weeks of treatment, etanercept reduced the responding patients’ auditory threshold from 55.7 ± 2.0 to 25.36 ± 1.2 dB.

Discussion

Using in vivo and in vitro models, this investigation has demonstrated that TNF-α induces a proconstrictive state throughout the cochlear microcirculation. In accordance with our overall hypothesis, TNF-α caused a rapid reduction of blood flow within the guinea pig stria vascularis, which was associated with decreased capillary diameter. TNF-α has been shown to elicit vasoconstriction in both human mammary arteries and mouse cerebral arteries: our in vitro measurements in spiral ligament capillaries confirmed that the TNF-α–stimulated reduction in capillary diameter was, in fact, an active constriction, rather than a passive collapse of the capillaries.

It is therefore tempting to speculate that microregional TNF-α production could compromise blood flow in specific regions along the length of the cochlea, which could give rise to hearing loss in distinct frequency ranges.

In addition to stimulating vasoconstriction within the distal cochlear microcirculation, TNF-α also augmented calcium sensitivity (that is, a proconstrictive state) in the more proximal gerbil SMA. Etanercept was able to block the TNF-α–stimulated augmentation of calcium sensitivity in control SMAs but not in those expressing the dominant-negative Sk1 mutant Sk1G82D (maximal diameter [diamax] of control = 90 ± 7 μm, n = 7; diamax of Sk1G82D = 92 ± 7 μm; n = 7). The effects of the chemical inhibition of Sk (by DMS, 3 μmol/L, 30 minutes) were similar to that of Sk1G82D expression: it reduced resting calcium sensitivity and prevented the calcium sensitivity increase after subsequent application of TNF-α (diamax = 87 ± 9 μm, n = 7 paired observations). DMS also reversed the TNF-α–stimulated enhancement of SMA calcium sensitivity (diamax = 86 ± 4 μm, n = 5 paired observations). F, TNF-α failed to increase calcium sensitivity in SMAs expressing the nonphosphorylatable, but catalytically active, Sk1 mutant Sk1S225A. *Denotes a significant difference (P < 0.05) in the dose-response relations.
The lack of effect of TB2-Sk1 expression in the SMA, and systemic pressures.16 The indiscriminate interruption of S1P signaling could therefore compromise this important function and lead to excessive pressure within the inner ear microcirculation: such a mechanism may explain why the S1P2 receptor–knockout mouse is deaf as a result of structural damage to the cochlear microcirculation and strial tissue.16 Because the SMA’s resistance-artery function appears to be dependent on constitutive S1P synthesis, interventions that target the S1P2 receptor (JTE013) and/or S1P synthesis (DMS) may not represent viable clinical strategies to counter the tone-enhancing effects of TNF-α.

We therefore assessed the effect of a catalytically active Sk1 mutant that lacks the ERK1/2 phosphorylation motif (Sk1S225A) necessary for TNF-α-stimulated activation of the enzyme.23 Remarkably, expression of Sk1S225A abolished the effect of TNF-α but retained a significant degree of calcium sensitivity. This identified the phosphorylation-dependent activation of Sk1 as a more precise molecular target for selective therapeutic approaches aiming to reduce the deleterious vascular effects of TNF-α. The fact that ERK1/2-dependent Sk1 phosphorylation integrates several inflammatory cytokine inputs (for example, interleukins 1β and -6)29,30 highlights the additional potential of this suggested therapeutic approach as a more general means to interfere with the microvascular effects of inflammatory processes. Pitson and coworkers25 have proposed that TNF-α–mediated Sk1 activation requires direct multiplexing with the TNF-α receptor, via an interaction with TNF-α receptor–associated factor 2. The lack of effect of TB2-Sk1 expression in the SMA, however, suggests that the TNF-α–mediated increase in calcium sensitivity relies on ERK1/2 activation downstream of the TNF-α receptor.

Patients who responded to etanercept displayed a functional hearing-recovery time course that could be tightly fitted to a single exponential function possessing an average time constant of 1.5 weeks. We acknowledge that the patient group was small; however, the time course, combined with low interpatient variability, suggests a single common mechanism underlying the benefit of etanercept treatment (that is, sequestration of TNF-α). The time course of hearing recovery, ~10 to 15 days, is consistent with the combined time for etanercept to reach effective plasma levels (~2 days),31 the normalization of blood flow (rather immediate, once therapeutically relevant concentrations of etanercept are established within the vascular wall), and for the endocochlear potential to be restored after oxygen deprivation (~7 days).32 Although none of these individual clinical observations directly links hearing loss to reductions in cochlear blood flow, the cumulative evidence provided from the human and animal data in this investigation supports a strong vascular component in the pathogenesis of hearing loss. Although we cannot exclude direct effects of TNF-α on hair cells (for example, apoptosis),33 the reversibility shown in Figure 6 is more consistent with a vascularly based mechanism.

In summary, our data indicate that TNF-α induces a proconstrictive state at all levels of the cochlear microcirculation via activation of S1P signaling: therefore, any pathology linked to the release of TNF-α (infection, autoimmune disorders, systemic inflammatory responses, etc) has the potential to cause vascularly based, ischemic hearing loss. In this scenario, failure of hearing transduction is secondary to ischemia in the inner ear, events that result from increased resistance to cochlear blood flow due to vasoconstriction of the SMA and cochlear microcirculation. As a result, this investigation integrates a subgroup of hearing loss cases into the family of ischemic vascular pathologies, with immediate implications related to risk stratification, diagnosis, and treatment.

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S.-S.B. and J.V.-B. dedicate this study to their mothers, who have been afflicted with sensorineural hearing loss.

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Disclosures
None.
References

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SUPPLEMENTAL MATERIALS AND METHODS

Materials
Sphingosine-1-phosphate (S1P) was purchased from Biomol (Hamburg, Germany), JTE013 from Tocris Bioscience (Ellsville, USA) and tumour necrosis factor α (TNFα) from Sigma-Aldrich (St. Louis, USA). The MOPS-buffered salt solution contained [mmol/L]: NaCl 145, KCl 4.7, CaCl₂ 3.0, MgSO₄ •7H₂O 1.17, NaH₂PO₄ •2H₂O 1.2, pyruvate 2.0, EDTA 0.02, MOPS (3-morpholinopropanesulfonic acid) 3.0, and glucose 5.0. Phosphate-buffered saline contained [mmol/L] 150 NaCl, 1.6 K₂HPO₄, 0.4 KH₂PO₄, 0.7 CaCl₂, 1 MgSO₄ and 5.0 glucose. Culture medium was composed of Leibovitz L-15 base medium supplemented with 15% fetal calf serum (FCS), 0.4µmol/L L-glutamine, 20000U/L penicillin and 20mg/L streptomycin. All buffer and culture medium components were purchased from Sigma-Aldrich. The plasmids encoding the sphingosine kinase 1 (Sk1) constructs (Sk1G82D, Sk1S225A, Sk1-GFP) have been previously described ¹². The TB2-Sk1 construct was prepared using site-directed mutagenesis as previously described ³ and confirmed by sequencing. Ketamine (Ketavet®; Parke-Davis, Berlin, Germany) and Xylazine (Rompun®; Bayer, Leverkusen, Germany) were used to anesthetize guinea pigs for intravital microscopy experiments.

Experimental Procedures
This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols complied with Canadian, American and German federal animal protection laws and were approved by the Institutional Animal Care and Use Committees at The University of Toronto, Toronto, Canada; Kansas State University, Manhattan, USA; and Ludwig-Maximilians University, Munich, Germany.

Intravital fluorescence microscopy of the cochlear microcirculation
The guinea pig cochlear window preparation has been described previously ⁴. Male albino Hartley guinea pigs (250-400g) that were anesthetized with i.p. injections of Ketamine/Xylazine (initial dose of 85mg/kg / 8.5mg/kg and supplemented 42.5mg/kg / 4.25mg/kg every 45 minutes thereafter). The core body temperature of anesthetized animals was kept at 38±1°C using a thermostatically controlled heating pad. A left femoral artery polyethylene catheter (PE50) served to monitor systemic blood pressure and obtain blood sample for arterial blood gas and serum electrolyte evaluation; intravenous injections were applied through a right external jugular
vein catheter. Heart rate and pulse oxygenation of the spontaneously breathing animals were continuously monitored.

The mastoid bulla was exposed and removed through a postauricular approach to the right ear. To provide better access to the cochlea, the tympanic membrane, the posterior annulus, and the tensor tympani tendon were cut and all middle ear ossicles sacrificed. Mucosa and mucosal vessels overlying the second and the third cochlear turns were gently wiped off by a piece of gel foam. A small rectangular window (0.2 x 0.3 mm in size) was cut over the convex part of the second turn and a small intact piece of bone was removed from the cochlea. The cochlear bony wall was elevated without traumatizing the underlying spiral ligament and stria vascularis to expose blood vessels in these tissues.

Intravital microscopy was performed using a modified Zeiss microscope (Axiotech Vario; Zeiss, Goettingen, Germany) and intravenously injected fluorescein isothiocyanate (FITC)-labelled dextran (MW 500,000; 0.05-0.1 ml of a 5% solution in 9% NaCl) to visualize the cochlear microcirculation. FITC was excited by epi-illumination (100 W mercury lamp) and observed via specific fluorescence filter blocks (excitation 450-490 nm, emission ≥515 nm). The area of interest within the cochlear window was magnified using a long distance 20x objective (Olympus SLMPlan). Images were acquired using a SIT video camera (C2400-08; Hamamatsu, Herrsching, Germany) and digitally recorded (AY-DV124AMQ; Panasonic, NJ, USA) for subsequent off-line analysis of vessel diameter and blood flow (Cap Image analysis system; Zeintl, Heidelberg, Germany). For each treatment group (i.e., TNFα and TNFα+JTE013), parameters were analyzed using a total of 6 capillaries from 3 guinea pigs.

**In vitro measurements of capillary diameter**

The isolation of gerbil spiral ligament capillary beds has been previously described. Gerbils were euthanized and then decapitated. The temporal bones were removed from the head and opened. The spiral ligament was dissected from the cochlea and the *stria vascularis* was removed. The isolated spiral ligament was transferred to a bath chamber as a flat sheet, where it was then continuously superfused with PBS (2.5 bath volumes exchanged each second). Because direct measurement of capillary diameter yields a poor signal-to-noise ratio, an indirect method, based on the measurement of red blood cell (RBC) movement with the capillary lumen, was utilized. RBCs trapped within the capillary lumen are perfect markers for luminal fluid
volume, since they do not stick to or disturb the vessel wall. Movement of luminal fluid and hence, RBCs, occurs in response to diameter changes and/or transendothelial fluid flux. Thus, under iso-osmotic conditions, the velocity of marker RBCs is a correlate measure of the vascular diameter. The precise model relating RBC movement to diameter is well-defined and has been described in detail previously.\textsuperscript{5,6}

Isolated capillaries were occluded on one end, opened on the other end, and RBCs trapped inside the lumen were visualized by laser-scanning microscopy (510 Meta, Carl Zeiss, Göttingen, Germany). The capillary lumen between the occluded end the RBC was assumed to be a cylinder of constant volume that undergoes proportional changes in diameter and length during vasoconstriction. The length of the capillary was determined using a hyperosmotic step (300-337mosM) that drives a defined water-flux across the capillary wall and is experimentally observed as RBCs travelling toward the occluder. Vasoconstriction elicits movement of RBCs towards the open end of the capillary.

A combined tracing illustrating RBC displacement following S1P stimulation is provided in Supplemental Figure 1. Representative time-lapse movies displaying capillary vasomotor responses are also provided as “Movie 1 - osmotic step.gif” and “Movie 2 - S1P.gif”

**In vitro measurements of vascular tone in the spiral modiolar artery**

The isolation of the spiral modiolar artery (SMA) has been previously described in detail.\textsuperscript{7,8} Gerbils were euthanized and then decapitated. The temporal bones were removed from the head, opened and placed in micro-dissection chamber containing ice-cold MOPS buffer. The otic capsule enclosing the cochlea was opened and the bone surrounding the modiolus was removed. The SMA was then gently removed from the eighth cranial nerve, taking care not to stretch the artery. Once isolated, the SMA was cut into ~1.5mm segments, cannulated and pressured hydrostatically to 30cmH\textsubscript{2}O. The vessel segments were maintained in culture medium for 24 hours, with continuous perfusion (culture medium) at a rate of 0.5ml/h. Transfection of genetic constructs (Sk1\textsuperscript{G82D}, Sk1\textsuperscript{S225A}, Sk1-GFP)\textsuperscript{1,2} was completed during the 24 hour vessel culture period using Effectene\textsuperscript{®} reagent (Qiagen; Hilden, Germany), according to the manufacturer’s instructions.

All functional experiments were performed at 37\textdegree C in MOPS buffer, as previously described.\textsuperscript{8} SMA viability was confirmed by testing its vasoconstrictor response to 3mmol/L Ca\textsuperscript{2+} under depolarizing conditions (125mmol/L KCl). All values of tone represent acute diameter
measurements that have been normalized: the values represent the magnitude of vessel constriction relative to maximal diameter. The computation of tone is as follows: tone (% of $\text{dia}_{\text{max}}$) = ($\text{dia}_{\text{max}} - \text{dia}_{\text{measured}}$)/$\text{dia}_{\text{max}}$ x 100. The apparent Ca$^{2+}$ sensitivity of the contractile apparatus was assessed as the relationship between microvascular tone and intracellular Ca$^{2+}$ levels. Intracellular Ca$^{2+}$ levels were adjusted by increasing extracellular Ca$^{2+}$ ($\text{Ca}^{2+}_{\text{ex}}$) from 0 to 10mmol/L under depolarizing conditions (120mmol/L K$^+$).

We have observed that the baseline level of calcium sensitivity can significantly fluctuate in separate batches of animals. We cannot explain why this occurs, however, the animal-to-animal variability within a batch of animals is generally quite consistent, which allows for clear statistical results with only 5-6 experiments per group. All experiments were therefore designed as paired experiments (i.e., pre/post-treatment) or, in the case of transfection experiments, all treatment groups within an experimental series were completed with the same batch of animals. Statistical comparisons across different experimental series were avoided.

*Description of human subjects included in etanercept treatment study*

The use of human subjects in this study conforms with the principles outlined in the Declaration of Helsinki $^9$ and was approved by the Human Studies Ethics Committee of the Technische Universität München; Munich, Germany (#2172/08).

A total of 12 adult patients presenting with the typical symptoms of SHL (with or without vertigo and/or symptoms of uni-/bi-lateral Meniere's disease) were identified and selected for treatment. As an inclusion requirement, patients showed either no or only a partial response to a conventional prednisolone treatment regime [high-dose, 500 mg/day i.v., for three days, followed by 16 days of weaning (starting at 100mg p.o. daily with 20mg dose reduction every 2 days and a further reduction to 10, 5, and 2.5mg/day)]. Patient exclusion criteria included: the presence of an underlying infection; a history of acute, chronic or recurrent serious infections; inadequate cardiac, pulmonary, renal or hepatic function; neurological disorders; elevated red blood cell count or C-reactive protein; and/or a history of malignancy. Female patients who were pregnant or breast-feeding were also excluded; non-menopausal female patients were required to use birth control for the duration of etanercept treatment.
Prior to enrolment, we obtained informed written consent for the off-label use of etanercept. Each patient’s cardiac, hepatic and renal functions were specifically assessed, as well as his/her overall health status, documented through an interview, physical examination, chest radiography, magnetic resonance imaging (MRI) of the brain and determination of several laboratory parameters (erythrocyte sedimentation rate (ESR), HsP70, white blood cell count, C-reactive protein (CRP), serum aspartate aminotransferase levels, syphilis serology, serum creatinine levels, glucose levels, antineutrophil cytoplasmic antibodies (ANCA) and antinuclear antibodies (ANA). Other medications for the treatment of symptoms caused by Meniere's disease (e.g., diuretics and sedatives) were halted for the duration of the study, as their actions could confound the results. Etanercept was self-administered subcutaneously twice a week for 12 weeks (25 mg; similar to the dose routinely used for the treatment of rheumatoid arthritis).

A composite of the patients included in the study is provided in Supplemental Table 1. A total of 19 ears (from 12 patients) with hearing loss were monitored; these were subcategorized into 9 cases of “acute” (symptoms <3 months) and 10 cases of “chronic” (symptoms present for >3 months) hearing loss, depending on when treatment for the condition was sought. Two patients reported adverse reactions to etanercept (neither patient displayed auditory improvement before discontinuation of treatment): the exclusion of their cases from data analysis reduced the respective pools to 8 acute and 7 chronic cases (i.e., 1 acute and 3 chronic hearing loss cases were excluded).

In 8 patients, 6 acute and 2 chronic cases of hearing loss was associated with systemic inflammatory diseases (i.e., rheumatoid arthritis, inflammatory bowel diseases, ankylosing spondylitis, diabetes mellitus type I, non-alcoholic fatty liver disease (NAFLD) and autoimmune hepatitis); non-immune mediated cochleovestibular disease (non-IMCVD), indicated by otologic symptoms, appeared to be the primary etiology in the other 5 patients. Five of the 12 patients presented with “unilateral” hearing loss (i.e., only in one ear). Patients with acute hearing loss (symptoms occurred for less than 3 months; 8 treated ears in 7 patients) were analyzed separately from those where the symptoms had become chronic (prevalent for more than 3 months; 7 treated ears in 5 patients), since permanent structural alterations in hearing transduction (i.e., death of sensory hair cells) may have occurred by this time. Our analysis excludes 2 patients that discontinued treatment due to adverse reactions.
Hearing function was assessed by pure-tone audiometry, speech reception threshold and word recognition. Pure-tone average (PTA) was performed from 9 frequencies between 250 and 6000Hz (250, 500, 750, 1000, 1500, 2000, 3000, 4000, and 6000Hz). Measurements were taken at 1, 2, 3, 4, 8, and 12 weeks post-initiation of etanercept treatment. Improvement of hearing was defined as an improvement of (i) sensorineural hearing from baseline (ii) ≥ 20 dB in the pure-tone air conduction thresholds in at least 3 of the tested frequencies and (iii) 15 dB at 2 consecutive frequencies.

SUPPLEMENTAL RESULTS

Specificity of JTE013

We have previously demonstrated that JTE013 inhibits S1P-stimulated vasoconstriction in the SMA and hamster resistance arteries. However, demonstrating that JTE013 specifically inhibits S1P$_2$ receptor-dependent responses in the SMA is rather difficult, because only two agents have been documented to evoke vasoconstriction in un-branched SMA segments (which we use in our experimental setup) to date: endothelin-1 (ET-1) and S1P. Although ET-1 stimulates SMA vasoconstriction, a portion of the response appears to be dependent on S1P (i.e., ET-1 stimulates S1P synthesis and consequently S1P$_2$ receptor dependent signaling pathways; unpublished observations): thus, ET-1 responses in the SMA could be partially inhibited by JTE013. In support of these observations, ET-1-dependent activation of Sk1 in smooth muscle cells has been demonstrated previously by others.

As an alternate means of assessing JTE013 specificity, we assessed the effect of JTE013 on norepinephrine responses in isolated hamster gracilis muscle resistance arteries. We used a norepinephrine concentration of 0.3μM, which elicits a sub-maximal constriction of the hamster resistance artery. We found no effect of JTE013 (pre-JTE013: 37±3%; post-JTE013: 38±2%; n=6 paired observations, P>0.05). We acknowledge that this control experiment was conducted using a different species and a vessel from a different vascular bed; however, in conjunction with our previous evidence that JTE013 inhibits S1P-dependent vasoconstriction in the same artery, this control experiment provides an indication that JTE013 is acting specifically at the 1μmol/L concentration.
SUPPLEMENTAL FIGURES

Figure 1: S1P stimulates a change in capillary diameter in spiral ligament capillaries
Shown are combined tracings (mean±SEM; n=9) of normalized red blood cell (RBC) displacements, a correlate of capillary diameter, in response to a hyperosmotic step (from 300 to 337 mosM; marked by “H”) and 100µmol/L S1P. Measurements were made in 1s intervals; error bars are given every 10s. The marker RBC moved toward the occluder when a water efflux was induced by the hyperosmotic challenge. In contrast, the marker RBC moved toward the open end, when the capillary constricted in response to S1P.
Movie 1: Laser-scanning microscopy of a capillary in the isolated spiral ligament: response to osmotic step.

The capillary was occluded on the left side and open on the right side. Increasing the osmolarity from 300 to 337 mosM caused movement of RBCs toward the occluder. Restoring the osmolarity from 337 to 300 mosM caused a similar movement of the RBCs to the open end. The original length of this sequence is 120 sec.

Movie 2: Laser-scanning microscopy of a capillary in the isolated spiral ligament: response to S1P.

The capillary was occluded on the left side and open on the right side. Addition of 100µmol/L sphingosine-1-phosphate (S1P) caused visible constriction of the capillary and movement of RBCs toward the open end. The original length of this sequence is 290 sec.
Figure 2:  S1P stimulates dose-dependent vasoconstriction of spiral ligament capillaries
S1P-stimulated reductions in capillary diameter were measured in an ex vivo preparation of capillary beds isolated from the spiral ligament of the cochlear lateral wall. The number of capillaries measured for each S1P concentration is indicated in parentheses. Using a sigmoid curve to fit the 4 data points, the EC$_{50}$ was approximated to be 4.7µmol/L.
### SUPPLEMENTAL TABLE

<table>
<thead>
<tr>
<th></th>
<th>sex</th>
<th>Hearing loss</th>
<th>Improvement during treatment (week 1-12)</th>
<th>Background diseases</th>
<th>ANCA ANA ds-DNS</th>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>♀</td>
<td>AHL, right ear</td>
<td>CR</td>
<td>Non-alcoholic fatty liver disease (NAFLD)</td>
<td>-</td>
<td></td>
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<tr>
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<td>♀</td>
<td>AHL, right ear CVD</td>
<td>CR</td>
<td>Diabetes mellitus Type I</td>
<td>-/+</td>
<td>Etanercept improved insulin resistance</td>
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<tr>
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<td>AHL, right ear</td>
<td>CR</td>
<td></td>
<td>-</td>
<td>Discontinued because of pregnancy after 14 days of treatment. CR within this time</td>
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<tr>
<td>4</td>
<td>♀</td>
<td>AHL, right ear</td>
<td>PR</td>
<td>Steatosis hepatitis</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>♀</td>
<td>AHL, both ears CVD</td>
<td>CR left ear PR right ear</td>
<td>Auto-immune hepatitis</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>♀</td>
<td>AHL, left ear CVD</td>
<td>NI</td>
<td>Immune-mediated CVD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>♀</td>
<td>AHL, left ear CHL, right ear CVD</td>
<td>PR, left ear (acute) NI, right ear (chronic)</td>
<td></td>
<td>-</td>
<td>AHL recovered completely within 1 year. CHL never improved.</td>
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<tr>
<td>8</td>
<td>♀</td>
<td>CHL, both ears</td>
<td>PR</td>
<td>HLA B27 assoc.; initial ankylosing spondylitis</td>
<td>+</td>
<td>Last hearing loss progression occurred more than a year prior to current treatment</td>
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<tr>
<td>9</td>
<td>♀</td>
<td>CHL, both ears</td>
<td>NI</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>♀</td>
<td>CHL, both ears CVD</td>
<td>NI</td>
<td></td>
<td>-</td>
<td></td>
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<td>11</td>
<td>♀</td>
<td>CHL, both ears CVD</td>
<td>NI after 2 weeks, treatment discontinued because of AR</td>
<td>Colitis ulcerosa</td>
<td>-</td>
<td>AR: rush at injection site</td>
</tr>
<tr>
<td>12</td>
<td>♀</td>
<td>AHL, left ear CHL, right ear</td>
<td>NI after 1 week, treatment discontinued because of AR</td>
<td>Hypothyroidism; Rheumatoid arthritis; RF: +</td>
<td>-</td>
<td>AR: rush at injection site &amp; headache.</td>
</tr>
</tbody>
</table>

**Table 1. Demographic characteristics of patients treated with etanercept**

Patients had a mean age of 44.4 ± 4.6 years. Involvement describes if patients had one or both ears affected. Acute hearing loss (AHL) was defined as a hearing loss that happened less than 3 months prior to enrolment. Otherwise hearing loss was termed chronic (CHL). Cochleovestibular disease was assumed in patients that had objectifiable signs of vestibular affection in addition to hearing loss.

**Abbreviations:** AR = Adverse Reactions; CR = Complete remission; PR = Partial remission; NI = No improvement; CVD = Cochleovestibular disorder; ANCA = Antineutrophil cytoplasmic antibody; ANA = Antinuclear antibody, ds-DNS = anti-dsDNA antibodies; RF = Rheumatoid factor.
SUPPLEMENTAL REFERENCES


