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Effects of TNF-alpha inhibition on inner ear microcirculation and hearing function after acute loud noise *in vivo*

(Effekte einer TNF-alpha-Inhibition auf die Mikrozirkulation und die Hörfunktion nach Lärmschädigung *in vivo*)

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Die Zusammenfassung

Bei einer akuten lärminduzierten Innenohrschwerhörigkeit kommt es zu einer erheblichen Mikrozirkulationsstörung im Bereich der *Stria vascularis*. Eine TNF-α-Inhibition kann eine Vasokonstriktion der Modiolusarterie im Innenohr durch die Inaktivierung der Sphingosinkinase-1 im S1P/$S1P_2$-Signaltransduktionsweg der vaskulären glatten Muskelzellen beseitigen. Desweiteren kontrolliert TNF-α eine NO-mediierte Vasodilatation im Innenohr. Aufgrund dieser Zusammenhänge erschien es denkbar, dass eine frühzeitige therapeutische TNF-α-Inhibition eine kochleäre Mikrozirkulationsstörung und eine Hörschwellenverschiebung nach akuter Lärmschädigung beseitigen könnte.

In der vorliegenden Arbeit wurde ein neues standardisiertes Tiermodell etabliert, um die akuten Effekte einer Lärmexposition auf die cochleäre Mikrozirkulation und die Hörfunktion mittels *In vivo*-Fluoreszenzmikroskopie und Hirnstammaudimetrie messen zu können. Hierzu wurde fluoreszierendes Dextran als Blutplasmamarker bei narkotisierten Meerschweinchen intravenös appliziert. Auf einem Ohr wurden die Cochlea und die *Stria vascularis* chirurgisch für die Mikroskopie dargestellt. Am kontralateralen Ohr wurde nach Lärmexposition beider Ohren (106 dB SPL, 30 min) zusätzlich die Hörschwelle gemessen. Kontrolltiere wurden nicht lärmexponiert. Im Gegensatz zu den Kontrolltieren sank nach Lärmexposition die cochleäre Blutfließgeschwindigkeit bei gleichbleibenden Durchmessern in Kapillarsegmenten der *Stria vascularis* um 44% bis zum Ende des Beobachtungszeitraumes (210 min). Gleichzeitig stieg die Hörschwelle um 23 dB an.

Unter Verwendung dieses Modells zur Evaluation eines Therapieansatzes, konnte gezeigt werden, dass eine einmalige systemische Gabe eines TNF-α-Inhibitors – *Etanercept* - die mikrozirkulatorische Störung erfolgreich beheben konnte und die Hörschwelle wiederhergestellt werden konnte. Bei Kontrolltieren, die mit Kochsalzlösung behandelt wurden, war die Erythrozytenfließgeschwindigkeit um 36% reduziert im Gegensatz zu Tieren unter TNF-α-Inhibition, bei denen sich bis zum Ende des Beobachtungzeitraums beinahe keine Reduktion (2,2%) zeigte. Gleichzeitig kam es bis zum Ende des Beobachtungzeitraums zu einem Anstieg der Hörschwelle um 20 dB SPL in Kontrolltieren und zu nahezu keinem Anstieg der Hörschwelle nach TNF-α-Inhibition.

Diese Daten belegen, dass einen TNF-α-Inhibition einen vielversprechenden Therapiesansatz bei einer akuten Larmschädigung des Innenohres darstellen könnte.

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ABBREVIATIONS

3D-FLAIR	Three-dimensional fluid-attenuated inversion recovery
8-iso-PGF2α	8-isoprostaglandin F2α
ABR	Evoked auditory brainstem response
AGEs	Advanced glycation end-products
AICA	Anterior inferior cerebellar artery
aka	Also known as
AS	Ankylosing spondylitis
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
BDNF	Brain-derived neurotrophic factor
°C	Degree celcius
Ca^{2+}	Calcium ions
CAT	Catalase
CCD	Charge-coupled device
cGK	Cyclic guanosine monophosphate-dependent protein kinase
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
Cl^-	Chloride ion
COX-1	Cycloxygenase-1
d	Diameter
dB	Decibels: unit for measuring the loudness of sound
dB SPL	Decibels sound pressure level
dBA	Decibels above reference noise
DNA	Deoxyribonucleic acid
DPOAE	Distortion product otoacoustic emissions
DV	Digital videocassettes
e.g.	exempli gratia, Latin for "for example"
eNOS	Endothelial nitric oxide synthase
FDA	Food and Drug Administration
FGF2	Fibroblast growth factor
FITC	Fluorescein isothiocyanato dextran
fvd	Functional vessel density
g	Grams
GDNF	Glial cell line-derived neurotrophic factor
GPX	Glutathione peroxidase
GSH	Glutathione
GSHE	Glutathione monoethyl ester
GS-SG	Glutathione disulfide: oxidized GSH
GTP	Guanosine triphospahte

h	Hours
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HES	Hydroxyethyl starch
ICAM-1	Intercellular adhesion molecule-1
IgG	Human immunoglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IVM	Intravital fluorescence microscopy
JNK	c-Jun N-terminal Kinase
JRA	Juvenile rheumatoid arthritis
K^+	Potassium ion
KATP	Adenosine 5'-triphosphate-sensitive potassium
kg	Kilograms
kHz	Kilohertz
KLH	Keyhole limpet hemocyanin
L	Litre
LOX	Lipoxygenase
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
µs	Micrometers
µs	Microseconds
MAP	Mean arterial pressure
mg	Milligrams
min	Minutes
MLC	Myosin light chains
mm	Millimeters
MRI	Magnetic resonance imaging
MW	Molecular weight
Na^+	Sodium ion
NAD(P)	Nicotinamide adenine dinucleotide
NAD(P)H oxidase	Nicotinamide adenine dinucleotide phosphate
NF-kappaB	Nuclear transcription factor-kappa B
NIHL	Noise-induced hearing loss
NKCC	Na^+,K^+,$2Cl^-$-cotransporter
nm	Nanometers
NMDA	*N*-methyl-d-aspartate
NO	Nitric oxide
NSS	Normal saline solution: 0.9% saline solution
NT-3	Neurotrophin-3

NTF	Neurotrophic factor
O_2	Oxygen
O_2^-	Superoxide radical
OAE	Otoacoustic emissions
ONIHL	Occupational noise-induced hearing loss
$ONOO^-$	Peroxynitrite
OSHA	Occupational Safety & Health Administration
PC	Personal computer
PE	Polyethylene l
PEG	Polyethylene glycol
PG	Prostaglandin
Q	Blood flow in vessel segments
RA	Rheumatoid arthritis
RAGE	Receptor for AGEs
Rho	Ras-related monomeric GTPase
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase-Polymerase chain reaction
S1P	Sphingosine-1-phosphate
$S1P_{1-5}$	Sphingosine-1-phosphate receptor subtypes
Sar1	1-Sarcosine
SIT	Silicon-intensified-target
Sk1	Sphingosine kinase 1
SMA	Spiral modiolar artery
SOD	Superoxide dismutase
SPL	Sound pressure level
sTNFRI	TNF soluble receptor type I
TEOAE	Transient evoked otoacoustic emissions
Thr8-AII	8-Threonine Angiotensin II
TNF	Tumor necrosis factor
TNFR	TNF-α receptor
US	United States of America
VIP	Vasoactive intestinal polypeptide
v_{RBC}	Red blood cell velocities
WHO	World Health Organization

I INTRODUCTION

It is known that loud noise can impair cochlear microcirculation and increase hearing thresholds. Nevertheless, it is still unclear how microcirculatory disturbance is linked to dysfunction. A further question regarding noise-induced hearing loss (NIHL) is, how to treat it. Under the assumption of impaired cochlear blood flow, agents that promote cochlear microcirculation should be able to prevent and/or recover hearing function in acute NIHL. Among several suggested vasoactive drugs, tumor necrosis factor (TNF) inhibitors may be a promising one that can restore hearing function by promoting cochlear blood flow through the signaling pathway between TNF-α and sphingosine kinase 1 (Sk1) on spiral modiolar artery (SMA) tone regulation. It was the aim of this study to (1) firstly establish a new experimental NIHL model for *in vivo* analysis of cochlear microcirculation and hearing function, and (2) evaluate the effects of etanercept, a TNF-α inhibitor, on cochlear microcirculation and hearing function after loud noise exposure.

1. Cochlear microcirculation and hearing function after loud noise exposure

Inner ear structures such as hair cells and the supporting cells are tissues that demand high metabolic energy. Under normal circumstances, proper homeostasis is maintained by regular blood flow from the labyrinthine artery to the stria vascularis. When cochlear blood flow is impaired by any kind of injury such as noise, vascular pathologies or other cochlear disorders; sensorineural hearing loss can subsequently occur, possibly due to ischemia.

Before investigating in detail how cochlear microcirculation is linked to hearing function, it is important to understand basic mechanism of hearing, anatomy of the cochlea, arterial blood supply, as well as essential functions of the stria vascularis. In this section, these topics are thoroughly reviewed, and a comparison of different techniques used in cochlear microcirculatory evaluation is added. Multiple mechanisms that cause NIHL were gathered and described thereafter. However, the main point of this work focused on reduced cochlear blood flow in NIHL and possible therapeutic strategies.

1.1 Mechanisms of hearing

After passage of the resonance phase in the external ear canal, sound waves are converted to mechanical movements by vibration of the tympanic membrane. Vibrations of the tympanic membrane are transmitted across the middle ear cavity, through three little ossicles; malleus, incus, and stapes. Thereby, sound is amplified and transmitted to the inner ear through the oval window membrane which is connected to the stapes footplate. Movement of the stapes generates pressure waves of the fluid inside the cochlea. Meanwhile, the round window acts as a pressure valve, bulging outward as pressure in the inner ear rises. Sound amplification is caused by hydraulic action of the tympanic membrane transferred to the stapes bone (= a function of the surface area) and, to a lesser extent, by lever action from all ossicles. Inside the cochlea, fluid waves evoke membrane vibrations that displace the hair cells' sterocilia, resulting in generation of electrical impulses.

There are two types of hair cells, inner and outer hair cells. The inner hair cell is crucial for acoustic signal transduction because it provides electrical input to the brain via a release of neurotransmitter glutamate. It is considered as a true sensory receptor for hearing perception. On the contrary, outer hair cells do not send direct afferent fibers to brain but increase sensitivity of hearing by amplifying the acoustic vibration in an electro-mechanical feedback to basilar membrane. The increase movement of the basilar membrane following the outer hair cells activation is known as cochlear amplifier, a function that improves hearing sensitivity and frequency discrimination [Gold T, 1948].

Sohmer [Sohmer H, 1997] has explained that there are (1) mechano-electrical transduction and (2) electro-mechanical transduction as consequences following the displacement of basilar membrane. The initial stage hereby, occurs after mechanical displacement of stereocilia opened ion channels (mechanotransducer –MET channels) at the tip of stereocilia, is called 'mechano-electrical transduction'. In both inner and outer hair cells, this transduction leads to the receptor potential that facilitates the neurotransmitter release and enhances the input to motor activity of outer hair cells, respectively. On the contrary, the 'electro-mechanical transduction' occurs when outer hair cells change their length and provide amplification of vibration in localized regions to the basilar membrane motion.

In inner hair cells, stereocilia are embedded in the tectorial membrane. When the basilar membrane vibrates, a shear force under the tectorial membrane deflects these sensitive stereocilia. The membrane-vibration pattern occurs from base of the cochlea to apex. A stretching of inner hair cell membranes leads to a change in transmembrane potential, and results in hair cell depolarization. This depolarization opens the voltage-gated calcium channels which allow calcium ions (Ca^{2+}) influx to the hair cell, thereby inducing a release of the neurotransmitter glutamate. Subsequently, activation of glutamate receptors on afferent nerve fibers elicits electrical transduction that propagates along the auditory nerve to the cerebral hemisphere.

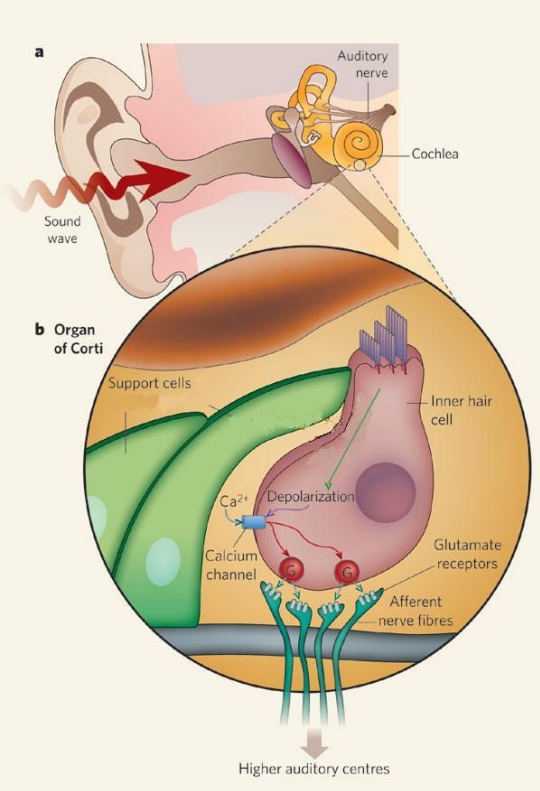

Figure 1 *The hearing process begins when (a) sound waves are transmitted from external and middle ear to the cochlea. (b) Vibration of the inner ear membranes leads to inner hair cell depolarization, opening calcium channels, and allows Ca^{2+} influx to trigger a release of glutamate, which activates the receptors on afferent auditory nerve fibers.*

*(Modified from Forsythe I.D.; Nature **450**: 43-44, 2007)*

The auditory (spiral) ganglion is the primary sensory ganglion for hearing. It is located within the bony confines of the cochlea. The auditory ganglion holds the neuronal cell bodies, therefore connecting the inner hair cells to cochlear nerve fibers. Multiple cochlear fibers converge to form the auditory nerve that runs within the internal auditory canal. After that, the auditory nerve leaves the temporal bone and runs to the lower brain, travels through the superior olivary complex, the lateral lemniscus, the inferior colliculus, and the medial geniculate body before ending in the auditory cortex of cerebral hemisphere [Luxon LM, 1981]. There nerve impulses are

transformed into recognizable sound. Additionally, at the level of cochlear nuclei, nerve fibers from each side of the ear split into two paths. The first path ascends straight up to the same side of auditory cortex in cerebral hemisphere, while the other crosses to the opposite side immediately after exiting the cochlear nuclei. As a result, each hemisphere receives information from both ears.

1.2 Cochlear anatomy

The cochlea, a bony structure with snail-like shape, is divided into three compartments by a delicate continuous membrane. The membrane is suspended within the cochlear bone, creating one compartment (scala media) sandwiched between the other two, scala vestibuli and scala tympani. Inside, it is filled with endocochlear fluid (endolymph for scala media, perilymph for scala vestibuli and tympani, respectively). The scala media is separated from the scala vestibuli by Reissner's membrane. Components of these two types of fluid are different. The composition of perilymph is similar to normal cerebrospinal fluid, whereas the endolymph resembles intracellular fluid. Endolymph contains low sodium and high potassium, which is essential for optimal function of hair cells.

The sensory organ of hearing is located within scala media. The Organ of Corti is a term for sensory receptors that lie on the basilar membrane and cells that hold the auditory hair cells. Thus, the organ of Corti consists of inner hair cell, outer hair cells, and supporting cells. The outer hair cells form three rows of sensory cells whilst the inner hair cells form only one.

As previously mentioned, these two types of hair cells behave in a distinct manner. The inner hair cell functions importantly as an auditory receptor whereas the outer hair cells are called the cochlear amplifier. They are also different in shapes, flask shaped for inner hair cells and cylindrical for outer hair cells; and in expression pattern of genes [Steel KP, 2001]. On the one hand, the inner hair cell is in contact to peripheral dendrites of the auditory nerve inside the spiral lamina. Therefore, hearing defects related to inner hair cells usually associate with molecules that affect the synaptic neurotransmitter release function of the calcium channel cells [Platzer J, 2000] or the protein otoferlin [Yasunaga S, 1999]. On the other hand, outer hair cells have localized motor function. They generate forces through the mechanism of somatic prestin motility and/or active bundle movement where the metabolic energy is used to amplify the mechanical responses of cochlear partition to sounds [Davis H; 1983, Dallos P, 2006]. Absence or depletion of the motor protein prestin [Zheng J, 2000] as well as the

molecules of potassium channels [Kubisch C, 1999; Dixon MJ, 1999; Delpire E, 1999] have been implicated as hearing defects by outer hair cells.

The outer wall of the cochlear duct is formed by periosteum and spiral ligament. Under the spiral ligament, there is a structure called stria vascularis which contains endolymph-producing cells and numerous capillaries.

1.3 Stria vascularis and hearing function

The stria vascularis is the area of special interest in this study. It is a part of the lateral cochlear wall which is extremely well-vascularized and very metabolically active. The stria vascularis is composed by three cell types: marginal, intermediate, and basal. Also, they can be divided into inner and outer epithelial layers. The inner epithelial layer consists of marginal cells whereas the basal and intermediate cells are included in the outer layer, which surfaces fibrocytes of the spiral ligament [Hinojosa R, 1966]. Between the two layers, there is a narrow interstitial space that contains numerous capillaries. These capillaries are the tiniest unit of cochlear vasculature and provide blood supply to the functional strial cells (Figure 2). The barrier between intrastrial fluid and blood plasma is comprised of endothelial cells that do not form fenestrae but are joined together by tight juctions [Jahnke K, 1980; Sakagami M, 1982].

It is understood that stria vascularis is crucial for hearing function because it controls the electrochemical composition of the endolymph. All epithelial layers in the stria vascularis are responsible for potassium ion (K^+) gradient by allowing ion transport across their membranes, but the appropriate ion composition is secreted to the endolymph by the marginal cells. According to Wangemann [Wangemann P, 2002], K^+ is the most important ion because it provides the major charge carrier for generating positive endocochlear potentials that cause hair cells depolarization, hence auditory nerve transduction. Mainly, the current flow is carried by the positive charges of K^+. To help the cochlea function properly, high concentrations of K^+ must be maintained by strial cells. After K^+ is secreted into endolymph, it enters hair cells through apical mechanosensitive channels [Pickles JO, 1992]. When the gated ion channel is opened, depolarization of the membrane potential occurs. This depolarization opens the voltage-gated Ca^{2+} channel and releases the neurotransmitter glutamate from the basal pole of inner hair cell [Dallos P, 1996].

Function of the strial cells in ion regulation

The endolymph contains only 2 mM sodium ion but 150 mM of K^+ [von Bekesy G, 1952]. In contrast, there is only 5 mM concentration of K^+ in the perilymph and in the blood plasma [Nin F, 2008]. The difference in electrochemical gradients results in endocochlear potential of +80 mV, compared to ~0 mV of electrical potential between plasma and perilymph, respectively (Figure 2B). Regulation of K^+ concentration is determined by various types of K^+ channels and transporters within multiple cell layers of stria vascularis. Two well-known channels are the Na^+,K^+-ATPase and the Na^+,K^+,$2Cl^-$-cotransporter (NKCC) which localize on membranes of the marginal cells [Nakazawa K, 1995; Crouch JJ, 1997]. In perilymph, K^+ is taken up by fibrocytes in the spiral ligament, and can pass by diffusion through basal and intermediate cells of the stria vascularis. In tight junctions between inner and outer epithelial layers, K^+ is taken up across the basolateral membrane of marginal cells via the Na^+,K^+-ATPase and the NKCC, and then secreted across the apical membrane to endolymph (Figure 2C).

In fact, not only potassium but also sodium helps maintaining electrical potential for hair cell transduction. Na^+ is expected to cycle between ion transporters within stria vascularis while K^+ is re-uptaken by different channels in the scala media [Jentsch TJ, 2000]. Studies in mice have demonstrated deafness and collapse of endolymphatic space as consequences of lacking K^+ channels [Flagella M, 1999; Rozengurt N, 2003]. Likewise, degeneration of stria vascularis including derangement of strial microvasculature, as well as diminished blood flow to this structure (despite normal capillary appearance), can result in a loss of cochlear electrical potential, and subsequent hearing loss [Seidman MD, 1999]. A correlation between stria vascularis and hearing function has also been emphasized by the histology of temporal bones from patients with presbyacusis, which showed significant loss of capillaries in cochlear lateral wall [Jennings CR, 2001].

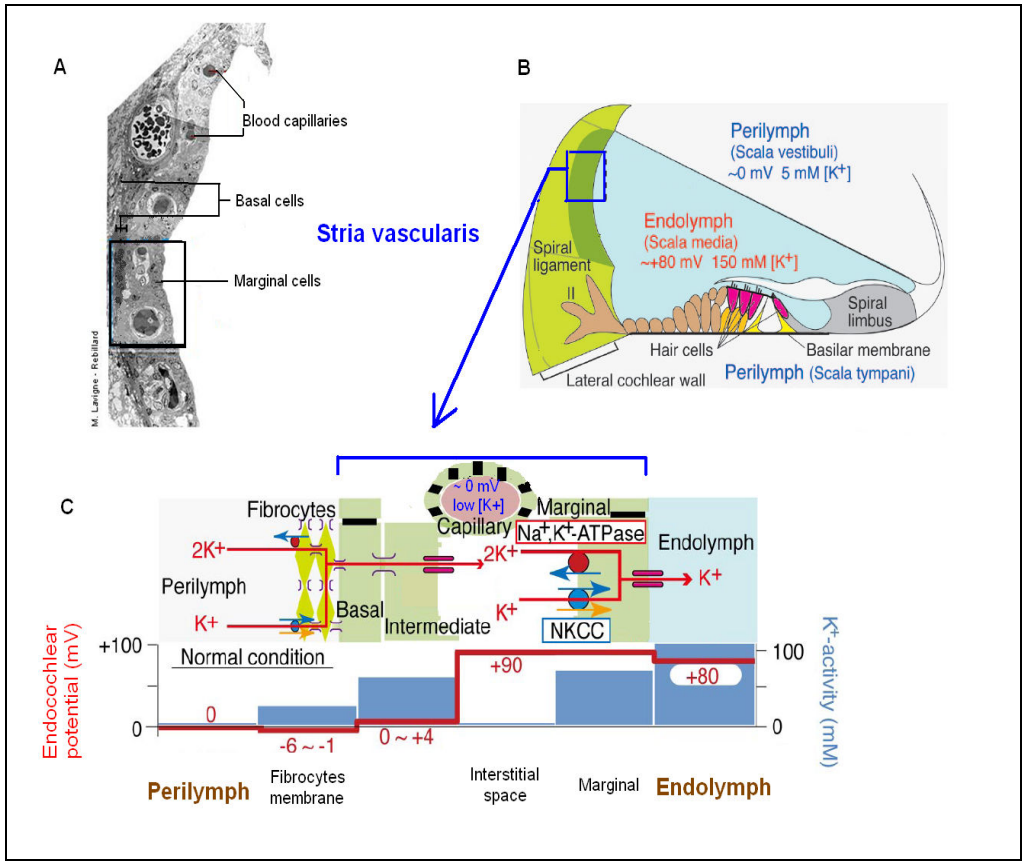

Figure 2 *Structure of the cochlea and stria vascularis. (A) Histological image of stria vascularis shows the layer of marginal cells, basal cells, and blood capillaries. (B) The difference of ion composition in the endolymph (Scala media) and perilymph (Scala vestibuli and tympani) results in a potential of ~ +80 mV . The area of stria vascularis is painted in dark green whereas the light green represents the spiral ligament. The box region (blue with arrow) is enlarged in A and C. (C) In the stria vascularis, capillaries are situated closely to the strial cells which contain active K^+ transport channels, for example, Na^+,K^+-ATPase and $Na^+,K^+,2Cl^-$-cotransporter (NKCC), and provide blood supply to the cells. When these cells function properly, the gradient of K^+ levels leads to differences of endocochlear potentials in perilymph, interstitial space, layers of stria vascularis, and endolymph, thereby providing driving force for sensory transduction. (Modified from Nin F. et al.; Proc Natl Acad Sci U S A* ***105****: 1751-6, 2008. and Rémy Pujol et al.; http://www.cochlea.org. University Montpellier and INSERM)*

1.4 Arterial blood supply of the cochlea

The cochlea receives its blood supply from a tight network of vessels embedded in the compact bone of the otic capsule. Blood supply to the cochlear apex, the second turn, and some regions of the basal turn rely exclusively on a functional end artery without collateral, the spiral modiolar artery (SMA). Meanwhile, the proximal part of the basal turn is fed by the cochlear branch of the vestibulocochlear artery [Mom T, 2005]. Both SMA and vestibulocochlear artery are the branches of common cochlear artery (see Figure 3). The vestibular branch of vestibulocochlear artery then runs toward the vestibule.

The common cochlear artery is originally derived from labyrinthine artery near the site where the cochlear nerve penetrates into the cochlear modiolus. In most humans, the labyrinthine artery arises from the anterior inferior cerebellar artery (AICA) which is a branch of the basilar artery and vertebral artery respectively. However, the labyrinthine artery may arise directly from the basilar artery in some individuals.

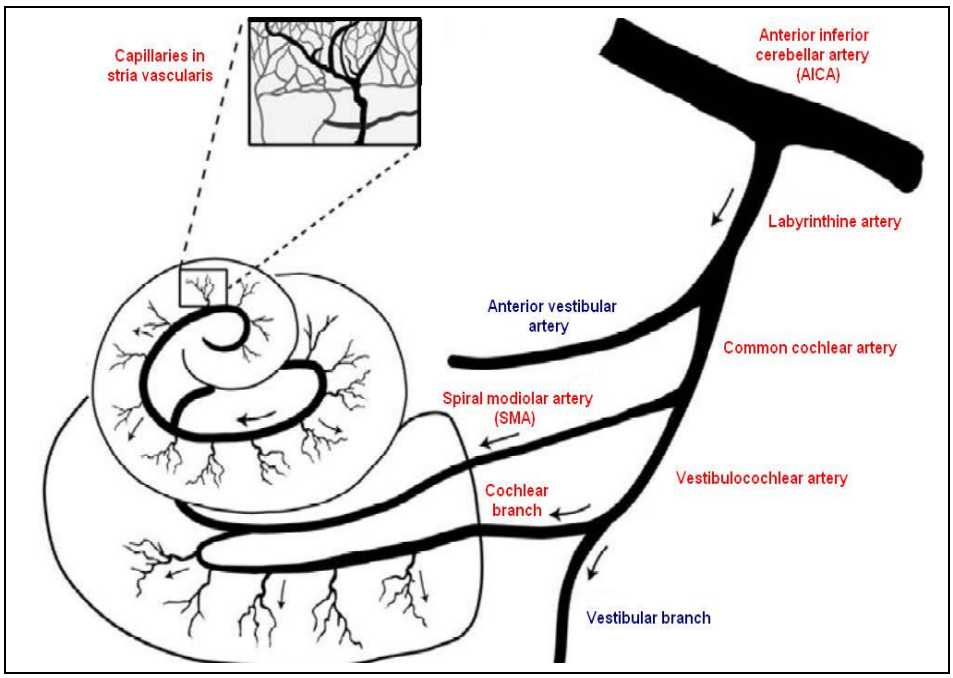

Figure 3 *Blood supply to the cochlea (Modified from Nakashima T et al.; Brain Research Reviews* ***42****: 17-28, 2003)*

According to Nakashima et al. [Nakashima T, 2003], the volume of cochlear blood flow is very small. It accounts approximately 1 x 10^{-4} of the total cardiac output in small animals like guinea pigs, and is estimated about 1 x 10^{-6} of the total cardiac output in humans. Although main arteries (SMA and cochlear branch of vestibulocochlear artery) that supply the cochlea are situated within the central portion of the cochlear vasculature area in the modiolous, distribution of cochlear blood flow is highest in the lateral portion where the stria vascularis and spiral ligament capillaries are located [Nakashima T, 1991].

1.5 Cochlear blood flow regulation

Sufficient blood flow to the cochlea is crucial for hearing function because the cochlea is a sensory organ that is highly sensitive to hypoxia. Among the cochlear feeding vessels, SMA seems to play a significant role in cochlear microcirculatory regulation [Wangemann P, 2002]. In addition to provide sufficient blood supply to most parts of the cochlea, SMA also protects the capillary beds in stria vascularis from systemic pressure in the labyrinthine artery [Scherer EQ, 2006].

In general, blood flow to the cochlea is appropriately maintained by a certain SMA smooth muscle tone (constriction and/or relaxation). Vasoconstriction reduces vessel diameter, thereby decreasing blood flow; whereas the relaxation of vascular smooth muscle dilates the vessel diameter and increases blood flow. The degree of vasoconstriction and/or dilatation of the SMA and its radiating arterioles must be adjusted properly to balance the energy demands of cochlear tissues.

To achieve proper regulation, vascular smooth muscle cells must integrate signals from multiple sources, for example, the signals from neuron networks around the vessels, from endothelial cells, as well as from the smooth muscle cells themselves. Many circulating hormones, local metabolic substances, and sympathetic innervation have been suggested as possible mechanisms that control cochlear blood flow. However, the hypothesis is most likely that the cochlear vascular tone is predominantly regulated by locally produced substances. Evidence from several animal experiments has confirmed the existence of an intrinsic autoregulation for cochlear blood flow [Brown JN, 1994; Ren T, 1994].

Among various regulatory mechanisms that have been revealed, the first well-established one is the adrenergic pathway [Spoendlin H, 1966; Laurikainen EA, 1994]. Vascular tone of the SMA is controlled by nerve

fibers from cervical sympathetic ganglia, as well as from the nerve fibers around AICA and basilar arteries. Stimulation of sympathetic nerves results in neurogenic and myogenic vasoconstriction [Wangemann P, 2005]. When the inner ear is under stress (for example, following loud noise exposure), the excessive sympathetic activity induces vasoconstriction and provokes local tissue ischemia, thereby resulting in sensorineural hearing loss. Reversely, sympathectomy can protect the ear from threshold shift following noise overstimulation [Borg E, 1982].

According to Carlisle et al. [Carlisle L, 1990] and Kitanishi [Kitanishi T, 1998], nerve fibers that innervate SMA and its branches contain both noradrenaline and vasoactive peptides (substance P, vasoactive intestinal polypeptide - VIP, calcitonin gene-related peptide - CGRP, and neuropeptide Y). Noradrenaline is a chemical transmitter that is characteristic for the sympathetic nervous system, whereas vasoactive peptides are polypeptides that can function concomitantly with either acetylcholine or noradrenaline depending on supplied nerve fibers. For instance, neuropeptide Y coexists with noradrenaline in perivascular sympathetic fibers while substance P and VIP act with acetylcholine upon perivascular parasympathetic activation [Ekblad E, 1984; Lundberg JM, 1991]. Accordingly, vasoconstriction is induced by stimulation of noradrenaline plus neuropeptide Y via α(1A)-adrenoceptors. Substance P might be involved in vasodilation [Tagawa T, 1997; Gruber DD, 1998]. As shown in a number of cochlear blood flow studies, administration of substance P to AICA can significantly increase cochlear blood flow [McLaren GM, 1993]. In contrast, administration of neuropeptide Y (plus noradrenaline) significantly reduces cochlear blood flow [Itou M, 2001]. It has also been acknowledged that endogenous substance P may interact with other vasodilators and vasoconstrictors within the cochlea via substance P-specific receptors to autoregulate the cochlear blood flow [McLaren GM, 1993]. However, neither presence of direct sympathetic innervation nor vasoactive peptides has been reported within the lateral wall of the cochlea [Nagura M, 2001].

The second mechanism that enormously contributes to cochlear blood flow regulation is the effects of nitric oxide (NO), a potent vasodilator. Unlike direct sympathetic innervation and vasoactive peptides, presence of NO is found in the cochlear lateral wall [Franz P, 1996; Yamane H, 1997; Michel O, 1999]. NO is an important endogenous substance that maintains blood flow by relaxing SMA vascular smooth muscle (vasodilatation) [Jiang ZG, 2004]. In endothelium, NO is produced from L-arginine by endothelial NO synthase (eNOS) [Palmer RM, 1987] in appropriate concentrations that protect the vessel from pathological changes (such as, from over-expression

of adhesion molecules, platelet degranulation and aggregation, leukocyte adhesion, and increases of blood pressure) [Albrecht EWJA, 2003; Wang-Rosenke Y, 2008]. But in an oxidative environment such as stress, another form of NOS is induced, called inducible NOS (iNOS). High production of NO by iNOS leads to higher possibility to react with superoxide and peroxynitrite formation, thereby resulting in cellular toxicity. Cumulatively, NO is known to participate in development of inflammation, neurotransmission, and oxidative stress reactions [Dawson TM, 1998; Wink DA, 1998; Guzik TJ, 2003; Korhonen R, 2005]. In the cochlea, accumulating evidence has confirmed the presence of eNOS staining in stria vascularis, spiral ligament, hair cells, and in nerve fibers of spiral ganglion [Franz P, 1996; Michel O, 1999].

NO production from endothelium is stimulated by hemodynamic forces, autacoids, hormones, and growth factors. Examples of eNOS activators include ATP, vascular endothelial growth factor, bradykinin, estrogen, angiopoietin, acetylcholine, and sphingosine-1-phosphate (S1P) [Fulton D, 2008]. Once NO diffuses into vascular smooth muscle layers, it reacts directly with a soluble form of guanylate cyclase and increases the level of cyclic guanosine monophosphate (cGMP). The cGMP is known as a second messenger that triggers muscle relaxation through activation of cGMP-dependent protein kinase (cGK). In turn, stimulation of cGK by cGMP leads to phosphorylation and reorganization of actin-myosin cytoskeleton in vascular smooth muscle cells [Heydrick S, 2000; Sessa WC, 2004] (Figure 4). The opening of cGMP-gated ion channels such as ATP-sensitive potassium (K_{ATP}) channels elicits a change in membrane potential (hyperpolarization), followed by inactivation of the voltage-gated L-type calcium channels and subsequent relaxation [Nelson MT, 1990; Si JQ, 2002; Jiang ZG, 2004].

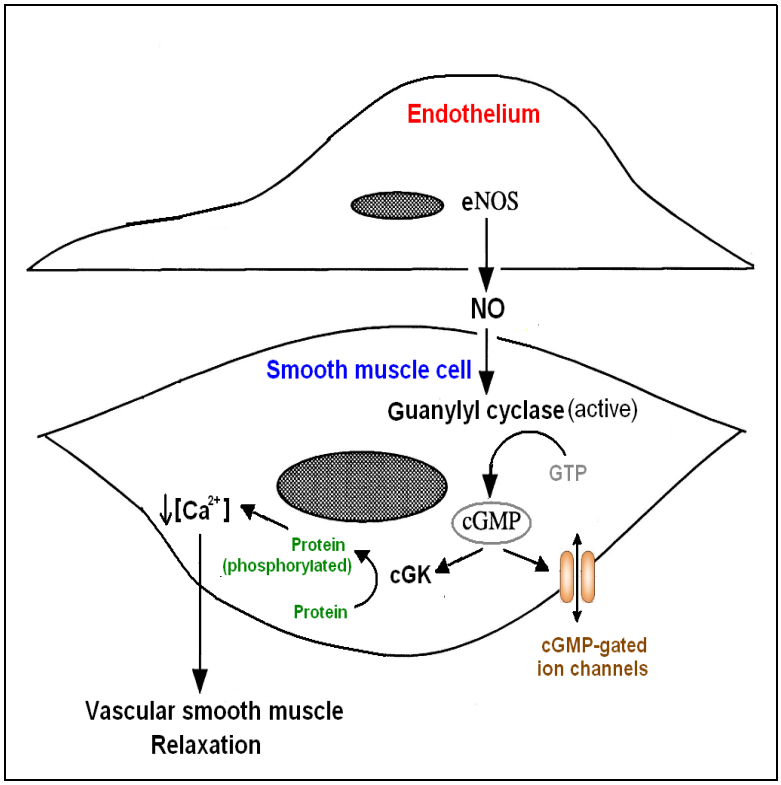

Figure 4 *Schematic illustration shows NO/cGMP signaling pathway in vascular smooth muscle* ***relaxation****. NO is produced by eNOS in endothelial cells, and then diffuses to the smooth muscle cells to activate soluble guanylyl cyclase, thereby converting guanosine triphosphate (GTP) to cGMP. Stimulation of cGK by cGMP leads to phosphorylation of protein substrates, enhancing K^+ efflux through cGMP-gated ion channels (e.g. K_{ATP} channel). In addition, intracellular calcium is reduced and relaxation of vascular smooth muscle finally occurs. (Modified from Travis W.H. et al.; Circ Res* ***85****: 634-42, 1999 and Ghofrani H.A. et al.; Nat Rev Drug Discov* ***5****: 689-702, 2006)*

On the other hand, constriction of arterial vessels is regulated by a distinct intracellular network which functions exclusively with phosphorylation of myosin light chains (MLC). Specific stimulation to smooth muscle cell (e.g. activation of α(1A)-adrenoceptors) results in a rapid increase of intracellular Ca^{2+} which therefore binds to calmodulin and activates MLC kinase. In turn, phosphorylation of the regulatory MLC by MLC kinase allows myosin ATPase to function and the muscle to contract [Kamm KE, 1985; Somlyo AP, 1994; Pfitzer G, 2001]. However, there is also another signaling cascade that acts concomitantly with the calcium dependent pathway (Ca^{2+} /calmodin) to contract smooth muscle cells. The latter is known as the calcium independent one that increases Ca^{2+} sensitivity of

contraction by inactivation of MLC phosphatase. In this cascade, Rho kinase, an enzyme that is activated by the Ras-related monomeric GTPase (Rho), plays a crucial role since it inhibits the activity of MLC phosphatase and increases muscle contraction force at constant Ca^{2+} concentration [Somlyo AP, 2000; Fukata Y, 2001]. Therefore, in conclusion, Ca^{2+}/calmodulin-dependent MLC kinase mediated MLC phosphorylation is the key factor for triggering vascular smooth muscle contraction, while Rho / Rho kinase signaling on Ca^{2+} sensitization is important for the sustained contraction.

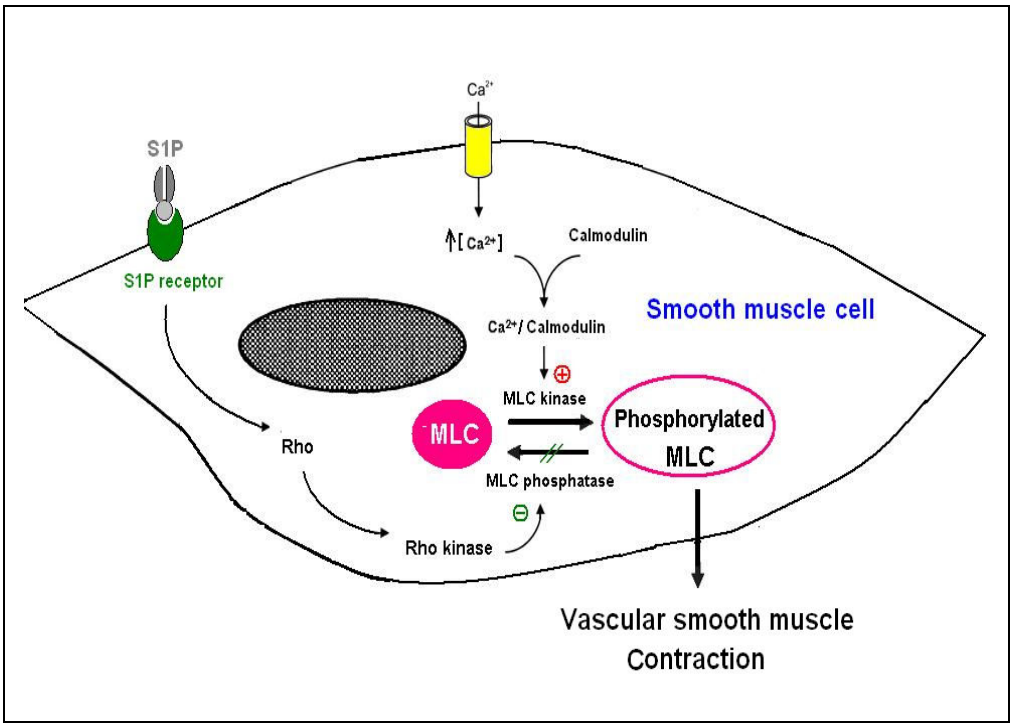

Figure 5 Contraction *of vascular smooth muscle appears to be regulated by intracellular network via activation and inactivation of the MLC phosphorylation. There are two primary mechanisms that concern this MLC regulation. The first mechanism is calcium dependent and activates MLC kinase via Ca^{2+}/calmodulin pathway to induce muscle contraction. The second mechanism is calcium independent that inhibits MLC phosphatase activity via the Rho/Rho kinase pathway, thereby increasing Ca^{2+} sensitivity of the contractile apparatus. In calcium dependent pathways, increased concentrations of intracellular Ca^{2+} may depend on the influx through both L-type and receptor-operated calcium channels.*

In association with the Rho / Rho kinase signaling cascade, sphingosine-1-phosphate (S1P) might be another significant local substance that participates in cochlear blood flow regulation. S1P might be responsible in

cochlear regulation as a vasoconstrictor for SMA. Increasing numbers of studies have accredited the action of S1P and its receptors on vascular tone [Ohmori T, 2003; Zhou H, 2004]. Loss of $S1P_2$ receptor in $S1P_2$ receptor-deficient mice lead to deafness upon vascular disturbance within the stria vascularis [Kono M, 2007].

S1P is released into the blood circulation by activation of particular physiological stimuli, such as proinflammatory cytokines (e.g. TNF-α) and growth factors (e.g. platelet-derived growth factor, vascular endothelial growth factor, and nerve growth factor) [Limaye V, 2008]. S1P is important as it is a ligand for a family of specific G-protein-coupled receptors ($S1P_{1-5}$) on the cell surface, which regulate multiple cellular functions, including vascular maturation, angiogenesis, cell growth, survival, cytoskeletal rearrangements, and cell motility [Spiegel S, 2003]. For cardiovascular activity, three distinct members of S1P receptors, $S1P_1$, $S1P_2$, and $S1P_3$, control homeostasis of the vascular system [Hemmings DG, 2004; Takuwa Y, 2008]. Three S1P receptors, $S1P_{1-3}$, are expressed in the SMA [Scherer EQ, 2006], particular $S1P_2$. $S1P_1$ receptors are largely expressed on endothelial cells [Igarashi J, 2008]. In contrast, $S1P_2$ and $S1P_3$ are expressed on vascular smooth muscle cells and can induce vascular bed vasoconstriction by Rho GTPase-dependent vasoconstriction [Bolz SS, 2003; Kono M, 2007; Salomone S, 2008]. According to Scherer et al., who applied S1P into isolated gerbil SMAs, vasoconstriction is caused by potent stimulating effect of S1P on the Rho/Rho kinase, which subsequently increases calcium sensitization of smooth muscle cells. However, in order to balance vascular tone, it has been discovered that S1P/$S1P_1$ receptor can also activate eNOS [De Palma C, 2006; Venkataraman K, 2008; Igarashi J, 2008], thereby inducing local NO production.

1.6 Studies of hearing function and cochlear microcirculation

Different approaches have been established to measure cochlear blood flow and the physiological processes related to cochlear microcirculation. Currently, laser Doppler measurements with either auditory brainstem response (ABR), distortion product otoacoustic emissions (DPOAE) or transient evoked otoacoustic emissions (TEOAE) were selected by most investigators to measure cochlear blood flow in accompanied with hearing function.

ABR has been applied to evaluate auditory function for a long time. Regarding to hearing levels, it allows measurement of threshold shifts after

loud noise exposure precisely and with high reproducibility regarding to the hearing level [Sliwinska-Kowalska M, 1992]. ABR is easily applied to animal subjects by placing needle electrodes under their skin. Among hearing measurements, ABR seems more sensitive to noise exposure duration than OAE [Fraenkel R, 2001]. For example, Fraenkel et al. have performed a study to investigate the effect of various noise exposure durations (3, 6, 9, 12, 15 and 21 days) on ABR, DPOAE, and TEOAE in rats. They found a significant increase of ABR thresholds in approximately 0.8 to 1.4 dB per day from linear regression analysis of the recorded responses and noise exposure durations. In contrast, TEOAE and DPOAE responses showed no similar dependence on noise duration. However, there is no clear advantage of mean DPOAE amplitude reduction over mean ABR threshold elevation in detection of temporary and permanent NIHL [Fraenkel R, 2003].

Despite numerous experiments with cochlear microcirculation, evidences that support the effectiveness of increased cochlear blood flow therapy for NIHL are still insufficient [Dengerink HA, 1984; Goldwin B, 1998; Lamm K, 1999; Lamm K, 2000]. Evaluation of the cochlear microcirculation is known to be difficult because of the complexity of the cochlea. In humans, the cochlea is hidden within temporal bone, and thus uneasy to access. Also, its size is too small so that images from angiography or MRI are not sufficient to visualize cochlear blood flow. Up to date, all approaches that have been used in studies of cochlear microcirculation are: (1) Intravital fluorescence microscopy (IVM), (2) laser Doppler flowmetry, (3) Microelectrode oxygen tension determination, (4) Cochlear blood measurements using microsphere techniques, (5) High resolution ultrasound and magnetic resonance imaging (MRI), and (6) Fluorescence microendoscopy.

Intravital fluorescence microscopy is an optical imaging method which offers the most reliable outcome owing to its ability to access blood flow within the individual single vessel directly. It was first performed in the 1980s to observe cochlear blood flow since the 1980s [Nuttall AL, 1987; Prazma J, 1989; LaRouere MJ, 1989]. However, this method became less popular than the laser Doppler techniques possibly because the later was easier to perform.

Laser Doppler flowmetry was first described for the study of cochlear microcirculation by Goodwin et al. (1984) [Goodwin PC, 1984]. This method offers good information about the flow and requires less invasive micro-surgery. However, it cannot resolve the blood flow at specific cochlear regions. Laser Doppler flowmetry provides only relative changes,

which is reported as ‘bulk flow’ rates, due to the uncertainty of the exact tissue volume measured at a certain timepoint. Furthermore, when using laser Doppler for examining the effect of loud noise, caution must be taken because the noise-induced artifact might mislead the blood flow reading by this instrument [Miller JM, 1990].

Oxygen microelectrode determination [Haupt H, 1993] provides a good dynamic performance, spatial resolution and accuracy of measurement. However, its results are related indirectly to cochlear blood flow as the oxygen utilization and supply are only one parameter in the measurements.

The techniques based on injection of either radioactive or labeled microspheres into blood circulation can express cochlear blood flow in absolute values at a certain timepoint in order to compare the differences of blood flow between cochlear regions [Larsen HC, 1985; Ohlsen A, 1994]. Nevertheless, these techniques depend on histological specimen of microsphere densities within vessels. This allows neither real-time imaging of blood flow dynamics nor the continuous acquisition of microcirculatory parameters.

Ultrasound and magnetic resonance imaging are less often used since they are unable to resolve blood flow directly. They are easy and non-invasive, but the current resolution of MRI is still not high enough for cochlear blood flow determination. However, both techniques have been used to reveal clinical correlations between hearing loss and intracranial circulatory disruption which is localized within the cochlea, particularly in patients with inner ear diseases. Doppler ultrasonography is sometimes used to detect cervical artery stenosis in patients with cochlear-vestibular symptoms [Gutmann R, 1993]. Up to date, three-dimensional fluid-attenuated inversion recovery (3D-FLAIR) MRI has been developed to detect high concentrations of protein or hemorrhage in inner ear of patients with sudden sensorineural hearing loss [Sugiura M, 2006].

The fluorescence microendoscopy is a novel *in vivo* imaging method, which affords resolution similar to that of the IVM and does not require extensive resection of surrounding structures [Monfared A, 2006]. It is claimed to be superior due to the ability to visualize cochlear microanatomy and blood flow simultaneously. Furthermore, it does not confound the cochlear blood flow signal with any superficial circulation and possibly preserving hearing function. However, this technique using endoscope probes is not yet sufficiently validated. Besides, the probe itself has rigidity which causes inability to bend along the curvature of the cochlear turns during the blood flow observation.

Accordingly, IVM alone can continuously follow the flow within single vessels, as well as accurately measure segmental blood flow in absolute values. The great advantages of this method are the abilities to:
(1) Measure the velocity of red blood cells in individual vessels
(2) Study the dynamics of microcirculatory changes
(3) Define the exact region of interest
(4) Investigate morphological and physiological changes.
It is well accepted that IVM studies of cochlear microcirculation have enabled visualization and measurement of blood flow directly and precisely.

Taken together, both ABR measurement and IVM are considered as the best methods to evaluate hearing function and cochlear microcirculation in animals.

1.7 Noise-induced hearing loss (NIHL)

Hearing impairment is a serious disability that has a huge impact on social life and the economic situation in individuals, families, and communities. The number of people affected by hearing loss had increased from 120 million in 1995 [World Health Organization, 1999] to 250 million worldwide in 2004 [Smith A, 2004]. Furthermore, according to the World Health Organization (WHO) in the year 2005, the estimated number of 278 million people worldwide was reported suffering from moderate to profound hearing loss in both ears.

NIHL is one of the most common causes of acquired sensorineural hearing loss, especially the hearing loss that is caused by hazardous noise at the workplace, called 'Occupational noise-induced hearing loss' (ONIHL). Nelson et al. [Nelson DI, 2005] indicate that occupational noise is an important risk factor of hearing loss in workers at most ages. It is accounted from 7% to 21% (averaging 16%) of the adult-onset hearing loss around the world, including developed countries.

NIHL is caused by either a one-time exposure to an intense impulse sound, such as an explosion, or by a continuous exposure to loud sound over an extended period of time, for example, exposure to 95-dB noise generated in a factory with noisy machine for more than 4 hours. Patients with NIHL usually present with sudden hearing loss and tinnitus following the exposure. As well as exposure to the excessive noise, exposure to

continuous noise can damage hair cells and supporting cells in the cochlea although the process occurs more gradually.

Exposure to impulse and continuous noise may cause either temporary or permanent hearing threshold shifts. Temporary threshold shifts which generally subside within 16 to 48 hours after exposure are observed in most cases of NIHL; however, a permanent threshold shift is sometimes present and affects seriously to the patients' quality of life. Occupational Safety & Health Administration (OSHA) of the US government has determined that exposure to loudness levels lower than 85 dBA continuously for an 8-hour workday is unlikely to cause harm. Likewise, in animals, there is evidence that high intensity noise of at-least-85 dB SPL for 6 hours can increase hearing threshold and decrease cochlear blood flow [Attanasio G, 2001].

1.8 Mechanism of NIHL

Many mechanisms have been postulated to explain the cochlear damage by noise exposure. It has long been believed that NIHL is caused by mechanical destruction of hair cells and supporting structures of the organ of Corti. Recently, growing knowledge supported the theory that an increase in mitochondrial free radical formation and reduced blood flow might be another explanation for NIHL.

Exposure to noise can be systematically divided into *exposure to a steady noise* or *exposure to an impulse noise*. The impulse noise is most likely to cause direct mechanical disruption to inner ear tissues, rather than steady noise. The sequelae of how steady noise induces hearing loss still remain unclear in spite of extensive investigations in the past. Anatomical changes of the inner ear structures possibly arise immediately after the impulse noise exposure including the edematous swelling and the detachment of stria vascularis from the spiral ligament [Ulehlová L, 1983; Hirose K, 2003].

The alterations are comprised with [Roland PS, 1997]
(1) Distortion of stereocilia, and/or
(2) Distortion of outer hair cells, and/or
(3) Distortion or Absence of the organ of Corti, and/or
(4) Rupture of Reissner's membrane.

How does NIHL relate to the changes of cochlear blood flow and hearing disability?

There are two main hypotheses that describe the mechanism of NIHL derived from steady noise. The first concept is based on similar effects as impulse noise inury: a direct mechanical trauma to the hair cells and supporting cells. The second concept is a new insight in inner ear pathophysiology: consequences following loud noise exposure are caused by metabolic exhaustion of the affected cells within the inner ear and an elevation of mitochondrial free radicals. In the latter, loud noise increases levels of reactive oxygen species (ROS) and damages cochlear tissues via (1) pathways of necrotic and apoptotic cell death [Henderson D, 2006], (2) excitotoxic neural swelling [Puel JL, 1998; Yamasoba T, 2005], and (3) intracochlear homeostasis alterations by reducing cochlear blood flow [Nuttall AL, 1999].

Reactive oxygen species (ROS)

Recently, many reports showed that oxygen-free radicals play an important role in several pathophysiologic processes. These oxygen-free radicals can modify and damage biologic systems. In NIHL, it is confirmed that high-intensity noise creates intense metabolic activity which increases mitochondrial free radical formation in the inner ear [Lim DJ, 1971; Yamane H, 1995; Ohlemiller KK, 1999]. ROS are in a more reactive state than molecular oxygen. These small molecules include ions, free radicals, and peroxides. They are known as byproducts during regular aerobic metabolism. Wolin has mentioned in a review [Wolin MS, 1996] that ROS result from biochemical reactions involving nitric oxide, ferrous iron, and other ions in the cytoplasm and the extracellular fluids. A primary ROS is superoxide radical (O_2^-) which is formed in a reaction catalysed by nicotinamide adenine dinucleotide phosphate (NAD(P)H oxidase).

$$\text{NAD(P)H} + H^+ + O_2^- \xrightleftharpoons{\textit{NAD(P)H oxidase}} \text{NAD(P)}^+ + H_2O_2$$

When superoxide anion interacts with enzyme superoxide dismutase, hydrogen peroxide (H_2O_2) is formed and will undergo further reactions to generate other ROS.

Sources of ROS are present dominantly in vascular walls. Enzymes that strongly participate in the control of vascular tone include arachidonic acid-metabolizing enzymes, eNOS, NAD(P)H oxidase, and xanthine oxidase. Wolin [Wolin MS, 1996] further reported that ROS are involved in

biochemical reactions of NO, which is a potential factor of vascular tone regulation, by converting NO to peroxynitrite ($ONOO^-$). The peroxinitrite can react with DNA, proteins, and lipids, leading to cellular damage. Moreover, after superoxide radicals transform NO into peroxynitrite, it is then converted to hydrogen peroxide. Correspondingly, a stimulation of the cytosolic form of guanylate cyclase is inhibited and the regulation of prostaglandins and the cGMP system are disturbed.

Under normal circumstances, every cell in the body has natural defense mechanisms to control levels of ROS. This defense is called *`Detoxifying´* which includes transformation (combine or catalyze) of ROS into less active forms. There are 3 types of natural antioxidants enzymes which play important roles in oxidative stress control. They are (1) superoxide dismutase (SOD) which converts superoxide anion to hydrogen peroxide (which however, is still toxic), (2) catalase (CAT) which changes hydrogen peroxide into water (H_2O) and oxygen (O_2), and (3) glutathione peroxidase (GPX) which transfers reactive electron from the peroxide to glutathione. These enzymes act in different reactions but aim similarly to maintain a balance for normal cell function.

$$2\ O_2^- \xrightarrow{(SOD)} H_2O_2$$

$$2\ H_2O_2 \xrightarrow{(CAT)} 2\ H_2O + O_2$$

$$2GSH + H_2O_2 \xrightarrow{(GPX)} GS{-}SG + 2H_2O$$

Cellular damages occur due to environmental stress when there is an imbalance between ROS production and the *Detoxifying*. Level of ROS can be dramatically increased after injury or in response to environmental stress.
Recent studies have indicated that ROS may be the common factor in NIHL and drug-induced hearing loss. Therefore, the use of antioxidants has been suggested for treatment in both situations [Henderson D, 1999].

As mentioned, a variety of studies have suggested that there is a causal relationship between ROS formation, hearing function and morphological damage after loud noise. In summary, considering NIHL, ROS can damage cochlear function in three ways:

(a) Direct injury to organ of Corti

In a study by Clerici et al. [Clerici WJ, 1995], a formation of bleb at the synaptic pole and a diminished cell length of cochlear outer hair cells are demonstrated as direct effects of ROS. Outer hair cells are more susceptible than inner hair cells to high intensity noise because outer hair cells are located in greater distance from the fulcrum of the basilar membrane than the inner hair cells. When the membrane vibrates extensively, following loud noise and more kinetic energy will apply to these outer cells, which therefore, are at greater risk of mechanical damage. It is also reported that the first row of outer hair cells is especially affected by noise [Spongr VP, 1992]. Furthermore, distinct structural properties of the organelle differ between outer and inner hair cells. In studies of ototoxicity, it seems that the outer hair cells appeared to be more susceptible to ototoxins. Furthermore, mitochondrial function of outer hair cells is impaired by ROS [Shi X, 2007]. Following noise exposure (120 dB SPL, 4 hours), formation of peroxynitrite is increased, while the mitochondrial membrane potential of the outer hair cells is reduced.

(b) Excitotoxic processes with destruction of neurons by excessive release of the neurotransmitter glutamate

It is known that glutamate induces toxicity in the neuronal system. From studies of stroke, it is fully accepted that ROS are major sources of cellular damage to the brain [Juurlink BH, 1997]. In NIHL, excessive amounts of glutamate is released after noise overstimulation, and causes auditory neuron damage by increased calcium accumulation or along oxidative cell death pathway [Atlante A, 2001; Le Prell CG, 2007]. On the other hand, administration of ROS scavengers or antioxidants can reduce glutamate-induced neuronal toxicity in retinal neurons and brain [Tastekin A, 2005; Matteucci A, 2005]. Puel et al. had observed the excitotoxicity at cochlear synapses after noise overstimulation [Puel JL, 1998]. Guinea pigs were exposed to loud noise, resulting in 80 dB hearing loss. A total disruption of synapses between inner hair cell and auditory nerve fibers was observed immediately within traumatized areas, possibly due to excessive release of glutamate.

(c) Reduction of blood flow to the cochlea

Noise-induced free radical formation might be a significant factor in cochlear blood flow reduction [Nuttall AL, 1999; Quirk WS, 1995]. In the

cochlear lateral wall where cochlear vasculature is located, multiple sources of ROS are found [Le Prell CG, 2007]. As mentioned before, NO is one of the most important substances that regulate cochlear blood flow by vasodilation. In NIHL, when ROS production is increased, large amounts of NO will be converted to peroxynitrite [Shi X, 2007], which is cytotoxic and can inhibit the stimulation of the cytosol form of guanylate cyclase, thus preventing vasorelaxing effects. Although there have been reports of increased NO production after noise-overstimulation, this excessive NO is eventually converted into peroxynitrite (NO competes with SOD for metabolizing cellular superoxide anions) [Wolin MS, 1996]. Hence, ROS such as superoxide anions can inhibit the cGMP-mediated pathway for smooth muscle relaxation, whereas hydrogen peroxide reacts directly with a soluble form of guanylate cyclase in vascular smooth muscle cells [Wolin MS, 1991].

Moreover, another potent vasoconstrictor which is also strongly related to ROS production plays a more significant role. According to Ohinata et al. [Ohinata Y, 2003] and Miller et al. [Miller JM, 2003], a level of 8-isoprostane-F2α (8-iso-PGF2α) formed in the organ of Corti significantly correlates to hearing threshold shift in the guinea pig cochlea after noise overexposure by reduction of cochlear blood flow. The 8-iso-PGF2α is one of the isoprostane compounds that are isomeric to cycloxygenase-derived prostaglandin [Taber DF, 1997] and formed in vivo by nonenzymatic free radical-induced peroxidation of arachidonic acid [Roberts LJ, 2000]. After being esterified to phospholipids by ROS which is a powerful oxidizer of lipids [Dormandy TL, 1989], 8-iso-PGF2α is then released in the free form and causes vasoconstriction.

Taken together, ROS can peroxidize lipids that exert formation of a potent vasoconstrictor, and link ROS production to the reduction of cochlear blood flow [Le Prell CG, 2007]. However, it is possible that 8-iso-PGF2α is not the only product that has an impact on cochlear microcirculation in NIHL. Other potent vasoconstrictors, such as sphingosine-1-phosphate, may also play its part in cochlear blood flow reduction. However, high expression of sphingosine-1-phosphate in NIHL has not yet been demonstrated.

In addition to vasoconstriction, stasis of blood cells in the cochlear lateral wall and spiral lamina vessels is recognized in noise-damaged cochleae [Nakai Y, 1988; Yamane H, 1991]. Both vasoconstriction and sludging of blood cells within the cochlear feeding vessels lead to impaired cochlear microcirculation. They interfere with the adequate supply of O_2 and nutrients, elimination of waste products, vascular permeability, and might cause ischemia in stria vascularis and other cochlear tissues [Quirk WS,

1995; Seidman MD, 1999; Hawkins JE 1971]. Regarding the fact that labyrinthine function closely relates to proper homeostasis, the decreased blood flow in strial capillaries can thus lead to hair cell dysfunction by dysbalancing K^+ ion and endocochlear potentials. Moreover, cellular and subcellular damage would occur, if oxygen supply continues to be inadequate. Further damage to mitochondrial DNA causes deficiencies in cellular metabolism and energy production [Seidman MD, 1999].

Cumulatively, ROS might alter vascular tone of the cochlear arterioles by
(1) Increased formation of vasoconstrictors
(2) Inhibition the NO/cyclic GMP-mediated pathways (suppression of natural vasodilatation)
(3) Direct injury to endothelial cells.

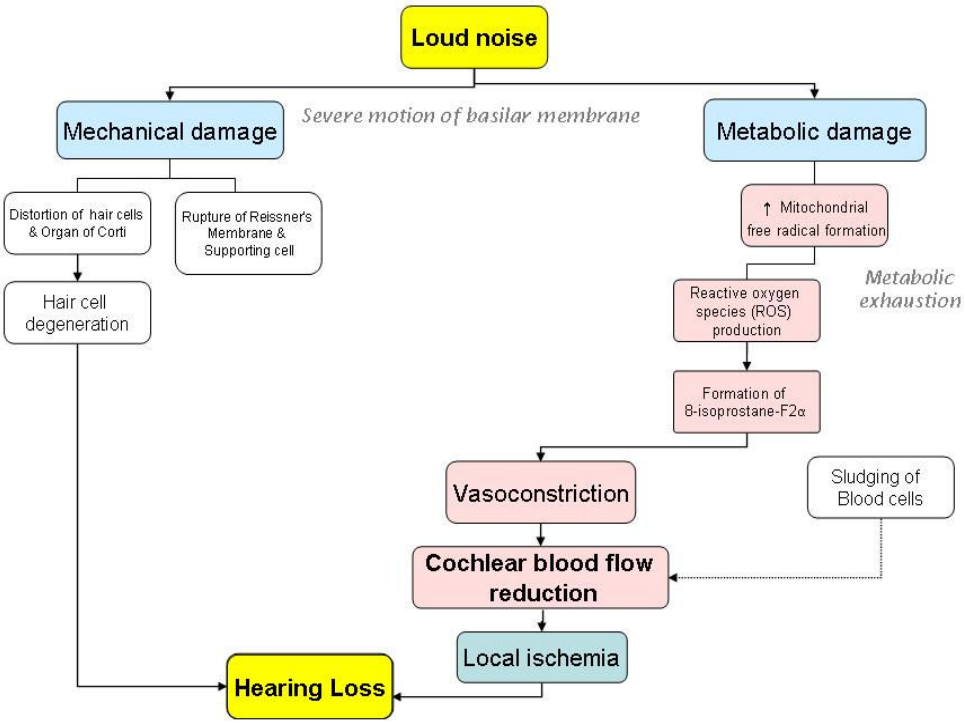

Figure 6 *Loud noise can lead to hearing loss along two pathways; mechanical and metabolic damages. In the later consequences, metabolic damage has a direct impact on cochlear blood flow. This scheme displays how local ischemia might cause NIHL. (Data based on a recent review by Le Prell et al. (2007) [Le Prell CG, 2007*])

1.9 Changes of cochlear blood flow and hearing threshold in NIHL

In 1971, Hawkins was among the first who showed an impact of loud noise on vascular morphology [Hawkins JE, 1971]. He noted that red blood cells are trapped within swollen endothelial of basilar membrane capillaries after exposure to an 8-hour wide-band noise at 118 to 120 dB SPL. Later on, many authors have studied and characterized several morphological changes as a response to loud noise in various cochlear vascular structures, either the basilar membrane, the spiral ligament, or the stria vascularis.

In summary, it has now been clearly demonstrated that cochlear microcirculation is altered when the inner ears are exposed to loud noise [Thorne PR, 1987; Scheibe F, 1993], [Lamm K, 1996; Attanasio G, 2001; Okamoto A, 1990; Dengerink HA, 1984] , [Quirk WS, 1995]. Thorne et al. [Thorne PR, 1987] demonstrated that loud noise (10-40 kHz, 103 and 110 dB SPL) causes reduction of cochlear blood flow by 25% compared to baseline values before noise exposure; whereas the lower intensity noise (90 dB) causes no change in cochlear blood flow over a period of 1 hour. Corresponding to Okamoto et al. [Okamoto A, 1992] and Scheibe et al. [Scheibe F, 1993], no change of blood flow is seen at lower intensities (80-105 dB SPL).

Different studies have demonstrated a varying degree of hearing threshold shift. A preliminary report by Sliwinska-Kowalska et al. [Sliwinska-Kowalska M, 1992] showed that ABR threshold shifts immediately after 2-h exposure to 112-dB noise, with various degrees of threshold shifts ranged from +25 to +65 dB. Attanasio et al. [Attanasio G, 2001] described that there is approximately 40 dB SPL of hearing loss after 24-h of loud noise exposure in chinchillas. Correspondingly, Yamasoba et al. [Yamasoba T, 2005] described that there is an elevation of ABR hearing threshold by 25 to 45 dB immediately after loud noise (3-h of 115 dB SPL, 4-kHz octave band). However, the reduction is lower than the loss of 48.9 $\pm$ 4.12 dB after 103 dB SPL (1-h exposure) duration reported by Thorne and Nuttall [Thorne PR, 1987].

Most authors agree that there is a prompt decrease in cochlear blood flow soon after the onset of noise exposure. Only Scheibe reported a significant decline in cochlear blood flow not before 40 min post-noise exposure [Scheibe F, 1993]. This decrease appears almost instantly after noise exposure and becomes progressively worse afterwards. In a study by Attanasio et al. [Attanasio G, 2001], a rapid increase of cochlear blood flow can subsequently be noticed within few days despite further consecutive

exposure to loud noise. This improvement of cochlear blood flow corresponds to hearing recovery. Nevertheless, all of these observations were derived from laser Doppler measurements.

Since the cochlea is an organ system with high energy requirements and the labyrinthine function is closely related to proper homeostasis, any reduction of cochlear blood flow can induce local ischemia, alter normal metabolic homeostasis, and finally result in a reduction of auditory sensitivity [Quirk WS, 1995; Seidman MD, 1999]. Nevertheless, a reduction of cochlear blood flow is not the only explanation for the changes in hearing function in NIHL. There is still no consensus on how microcirculatory disturbances are linked to hearing level changes of the inner ear.

2. Treatment of acute NIHL

In this study, a focus was made on the importance of cochlear blood flow on hearing function. As known, excessive noise can damage the inner ear and weaken hearing ability mostly by inducing a temporary threshold shift. Overstimulation of noise can cause permanent hearing impairment or deafness. Although NIHL is preventable, its extent is often unpredictable and not easy to treat. Thus far, there is no evidence based treatment for NIHL.

2.1 Multiple therapeutic interventions for NIHL

Various therapeutic interventions have been proposed for NIHL. These treatments target on different mechanisms which are theoretical causes of NIHL. Some pharmacological remedies can limit the damage from ROS or interrupt the apoptotic biochemical cascade to prevent the death of the irreplaceable hair cells. In order to prevent cellular damage and hair cells death, antioxidants as well as c-Jun N-terminal Kinase (JNK) inhibitors have been used to neutralize ROS and reduce hair cell apoptosis. Alternatively, other research groups have focused on vasoactive agents to increase cochlear blood flow and decrease cochlear ischemia.

Summary of therapeutic interventions used to treat or prevent NIHL

Therapeutic interventions	Examples [reference]
1) ROS inhibitors / scavengers such as antioxidants	
• Vitamin	Vitamin A, [Le Prell CG, 2007], Vitamin C [McFadden SL, 2005], and Vitamin E [Hou FX, 2005 ; Le Prell CG, 2007]
• Glutathione (GSH) pathway, which includes; a) GSH precursors	N-acetylcysteine (NAC) [Kopke RD, 2000 ; Lorito G, 2008], Resveratrol [Seidman M, 2003]
b) GSH monoethyl ester (GSHE)	GSHE [Ohinata Y, 2000]
c) GSH oxidase (GPx) - mimic	Ebselen [Pourbakht A, 2003 ; Kil J, 2007]
d) GSH repletion drugs	D-methionine (MET) [Kopke RD, 2002 ; Campbell KC, 2007]
• Allopurinol	Allopurinol [Seidman MD, 1993 ; Franzé A, 2003 ; Cassandro E, 2003]
• Superoxide dismutase (SOD)	Copper-zinc SOD [Cassandro E, 2003], SOD-polyethylene glycol (PEG) [Seidman MD, 1993]
• Magnesium	Magnesium [Attias J, 1994 ; Scheibe F, 2001; Attias J, 2004 ; Sendowski , 2006 ; Abaamrane L, 2009]
• Melatonin	Melatonin [Karlidağ T, 2002 ; Bas E, 2008]
2) Agents that reduce hair cell apoptosis by disrupting mitochondrial cell death pathway through peptide inhibition of JNK	D-JNKI-1 peptide [Wang J, 2007], AM-111 [Coleman JK, 2007 ; Suckfuell M, 2007], Retinoic acid [Shim HJ, 2009]
3) Agents that prevent neural degeneration	
a) via growth factors (also promote cell survival) • Exogenous NTF	Glial cell line-derived neurotrophic factor (GDNF) [Yamasoba T, 1999 ; Altschuler RA, 1999 , Shoji F, 2000] or brain-derived neurotrophic factor (BDNF) [Altschuler RA, 1999], neurotrophin-3 (NT-3) [Shoji F, 2000], neurotrophin-4/5 [Gillespie LN, 2004]
• Other growth factors	Basic fibroblast growth factor (FGF_2) [Zhai SQ, 2004 ; D'Sa C, 2007]
b) via inactivation of *N*-methyl-d-aspartate (NMDA) receptors on excitotoxicity (aka. Glutamate antagonist) • NMDA receptor antagonists	Carbamathione [Kopke RD, 2002], MK-801 [Duan M, 2000 ; Chen GD, 2001], Caroverine [Chen Z, 2004]

4) Vasoactive agents	
• Calcium channel blockers	Verapamil [Goldwyn BG, 1997]
• Specific angiotensin-II receptor antagonist (Sar 1, Thr8-AII)	Sarthran [Goldwin B, 1998]
• Corticosteroids	Prednisolone [Lamm K, 1998], Methyl prednisolone [Takahashi K, 1996 ; Tabuchi K, 2006], Dexamethasone [Takemura K, 2004]
• NSAIDS (only Cycloxygenase 1 (COX-1) inhibitors and Lipoxygenase (LOX) inhibitors	Diclofenac [Lamm K, 1998], Salicylate [Kopke RD, 2000], Indomethacin [Hoshino T, 2008]
• Hyperbaric oxygen therapy	Hyperbaric oxygen [Pilgramm M, 1985 ; Lamm K, 2000 ; Ylikoski J, 2008]
• Hyperoncotic hydrophilic hemodilutive plasma expander	Low-molecular-weight hydroxyethyl starch (HES) 70 or HES 200 [Pilgramm M, 1986 ; Lamm K, 2000]
• Low-molecular-weight dextran	Dextran 40 [Jakobs P, 1977]
• Others (Betahistine, Pentoxifylline, Tranexamic acid, Ginkgo biloba, Piracetam)	Betahistine [Lamm K, 2000] Pentoxifylline [Latoni J, 1996 ; Lamm K, 2000] Tranexamic acid [Tran YH, 2001] Ginkgo biloba [Stange G, 1975 ; Lamm K, 2000] Piracetam [Psillas G, 2008]

2.2 Vasoactive therapy

It is known that cochlear blood flow is reduced in several inner ear pathologies (e.g. infection, autoimmune diseases) including NIHL. Hence, in order to recover from these conditions, addition of vasoactive agents to the regular treatment has been proposed. In idiopathic sudden sensorineural hearing loss, it is recommended to use vasoactive agents to increase cochlear blood supply, improve tissue oxygenation and regain hearing function [Kohut RI, 1993]. However, similar conclusions have not yet been drawn for the treatment of NIHL although animal studies have already well demonstrated the effectiveness of such a treatment [Dengerink HA, 1984; Goldwin B, 1998; Lamm K, 1999; Lamm K, 2000]. Vasoactive agents experimentally can attenuate NIHL by dilating cochlear feeding arterioles and/or reducing vasoconstriction, thereby enhancing cochlear blood flow.

Up to date, various kinds of vasoactive agents have been proposed to treat NIHL. Some agents interact with local factors such as metabolic substances, or systemic factors such as circulating hormones and the sympathetic innervation to stimulate blood flow. Nevertheless, not all agents with vasodilating effects could be used. For instance, the calcium channel blockers did not always show protective effect against loud noise although they prevented vessel diameter from constriction and decreased

vascular permeability [Boettcher FA, 1996; Ison JR, 1997; Goldwyn BG, 1997]. In contrast, many of them that are not primarily used as cardiovascular vasodilators have been demonstrated to enhance cochlear blood flow effectively and protect hearing function, for example, corticosteroids, betahistine, and tranexamic acid.

Among them, HES seems to be the most effective blood flow promoter in NIHL, particularly when administered in combination with pentoxifylline [Lamm K, 2000]. Another vasoactive agent that is widely accepted to protect against NIHL is magnesium. In addition to its well-vasodilating effect, magnesium can also modulate calcium channel permeability, calcium influx into inner hair cells, and glutamate release. Recent studies have reported a high effectiveness of magnesium combined with antioxidant agents (vitamins A, C, E) in reducing both hearing loss and cell death prior to noise exposure [Le Prell CG, 2007]. The effectiveness of some drugs (e.g. ginkgo biloba, naftidrofuryl) is still controversial due to contradicting results from variable studies. If pathophysiology of metabolic damage from tissue ischemia is reversible, early use of vasoactive drugs might yield benefits in treatment of NIHL.

2.3 TNF-α expression and NIHL

For years, scientists have tried to link the immunological phenomena to noise injury. Recently, it has been acknowledged that inflammatory responses play a role in inner ear injury particularly in the early phase after over-exposure to noise [Miyao M, 2008]. For instance, infiltration of monocytes is observed in the histology of noise-damaged cochleae [Hirose K, 2005]. Besides, loud noise exposure can activate a cochlear immune response by upregulating cytokine expression and infiltration of circulating leukocytes [Tornabene SV, 2006; Fujioka M, 2006; Miyao M, 2008].

Based on data from the literature, major proinflammatory cytokines involved in the response to trauma include TNF-α, interleukin-1β (IL-1β), IL-6 and IL-8. These cytokines are produced from activated macrophages, monocytes, T cells, B cells, endothelial cells and fibroblasts, with additive or overlapping effects. Among them, TNF-α and IL-1β are the primary cytokines that are expressed in immune response and release various secondary cytokines, such as IL-6 and IL-8. Therefore, several inner ear studies have focused on the expression of proinflammatory cytokines. With regard to the lateral cochlear wall, it is reported that IL-1β is expressed by fibrocytes in the spiral ligament, whereas the expression of TNF-α is found in infiltrating inflammatory cells [Spicer SS, 1996; Satoh H, 2002].

Interesting studies that link TNF-α expression to cochlear inflammation are demonstrated by Satoh et al. (direct immunological stimulation) and Fujioka et al. (noise injury). During immune-mediated inflammatory response, Satoh et al. [Satoh H, 2002] discovered an expression of TNF-α in infiltrated white blood cells within 3 hours after keyhole limpet hemocyanin (KLH) injection. The level of TNF-α expression remained constant in scala tympani for further 48 hours. However, a similar effect occurred earlier and ended sooner in noise injury [Fujioka M, 2006]. As shown by Fujioka et al., a rapid rising of TNF-α level was observed immediately after noise exposure (124 dB SPL, 2 h) and even before IL-1β expression. The TNF-α level remained higher comparing with baseline values for at least 6 hours.

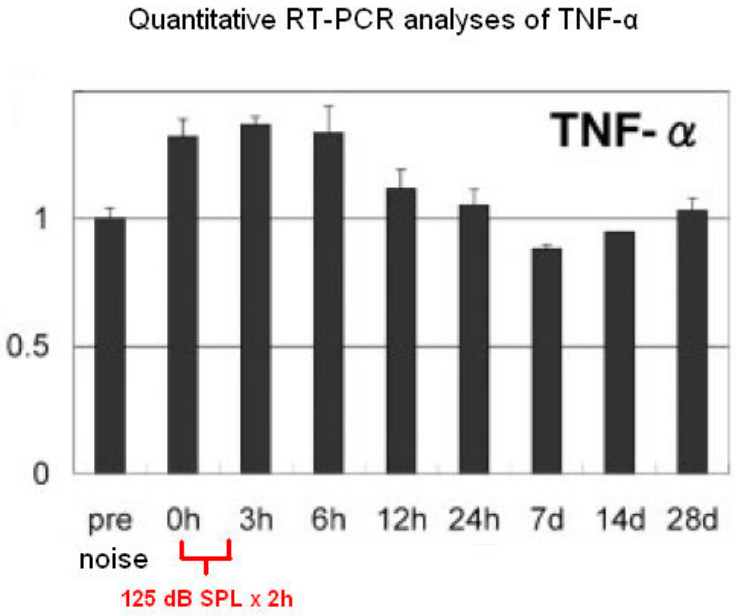

***Figure** 7 Quantitative RT-PCR analysis from rats' cochleae showed rapid increase of TNF-α level immediately after loud noise exposure [Fujioka M, 2006]. The vertical bar represents the relative ratio of (targent gene)/ (reference gene). TaqMan probes and 18S rRNA were used as a reference gene. (From Fujioka M.; J Neurosci Res **83**: 575-83, 2006)*

Accumulating evidence suggests TNF-α, a key mediator in local inflammatory responses, has the potential to impair vascular activity [Zhang H, 2009]. Although low levels of TNF-α help maintaining homeostasis, the elevated levels of TNF-α increase vascular permeability, thereby recruiting

inflammatory cells to the injury sites, as well as induce vasoconstriction and ROS production. Effects of TNF-α on vascular dysfunction have been closely looked at in various diseases - for instance, stroke [Hallenbeck JM, 2002], vascular aging [Bruunsgaard H, 2000; Csiszar A, 2007] - and some inner ear pathologies including autoimmune inner ear diseases [Staecker H, 2002; Wang X, 2003; Cohen S, 2005] and especially in NIHL [Zou J, 2005; Fujioka M, 2006]. Effects of TNF-α on cochlear vasculature are demonstrated by experimental models of immune-induced labyrinthitis where a rapid robust autoimmune response and hearing loss are observed. High numbers of inflammatory cells and rising levels of cytokines including TNF-α are expressed in the cochlear lateral wall [Chen MC, 1998; Satoh H, 2002]. Then, these inflammatory cells migrate from the systemic circulation to the scala tympani through the walls of spiral modiolar vein and its collecting venules [Harris JP, 1990; Fukuda S, 1992; Hoistad DL, 1998]. In conclusion, TNF-α is the earliest cytokine that is expressed in the cochlea in response to acute inflammation - including noise injury. This proinflammatory cytokine is able to amplify signals in several pathways leading to local inflammation, vascular dysfunction, tissue degradation, and cellular apoptosis.

2.4 Roles of TNF-α and Sphingosine-1-phosphate (S1P) on spiral modiolar artery (SMA) tone regulation

According to Zhang et al. [Zhang H, 2009], Advanced glycation end-products (AGEs)/receptor for AGEs (RAGE), Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and Nuclear transcription factor-χ B (NF-χB) increase the circulating amounts of TNF-α and/or local vascular TNF-α production. Increased TNF-α expression induces activation of NAD(P)H oxidase and production of ROS [Gao X, 2007]. Corresponding to Ungvari et al. [Ungvari Z, 2003] and Csiszar et al. [Csiszar A, 2007], exogenous recombinant TNF-α can elicit endothelial dysfunction, induce oxidative stress by up-regulation of inflammatory mediators and/or activation of NAD(P)H oxidase, as well as increase endothelial apoptosis and proinflammatory gene expression in carotid arteries of young rats.

The effects of TNF-α on SMA tone regulation could be explained by a relationship between the S1P/$S1P_2$ signaling in SMA smooth muscles cells and the fact that TNF-α is a potent activator for enzyme sphingosine kinase 1 (Sk1). As mentioned before, S1P is the bioactive lipid that acts as a potent messenger molecule. It is derived from ceramide in animal cells and then converted to sphingosine by enzyme ceramidase [Augé N, 2000]. Phosphorylation of sphingosine by Sk1 or Sk2 leads to the active form of

S1P. Sk1 is the enzyme that is strongly activated by TNF-α [Hanna AN, 2001]. Thus, presumably, S1P is released into the blood circulation by the activation of TNF-α. Then, S1P forms ligand bindings to the $S1P_2$ receptor, stimulating Rho/Rho kinase, and increasing calcium sensitization of the contractile apparatus through MLC phosphorylation in the inner ear vascular smooth muscle cells.

2.5 TNF-α inhibitors as a new treatment option for acute NIHL

Based on the aforementioned data in addition to the fact that TNF-α is the earliest proinflammatory cytokine in noise-overstimulation, we hypothesized that the therapy with TNF-α inhibitors may be a new treatement option for NIHL. It is possible that TNF-α inhibitors can restore hearing function from loud noise by acting as vasoactive agent.

There are at least three of TNF antagonists available nowadays, for example, etanercept, adalimumab, and infliximab. All of these drugs have anti-inflammatory properties by acting against TNF-α. Etanercept (Enbrel®) is a recombinant human protein which acts as a TNF-α receptor 2 (TNFR2) analog, whereas the adalimumab (Humira™) is a recombinant human IgG monoclonal antibody specific for human TNF-α. Infliximab (Remicade®) is a chimeric IgG monoclonal antibody, which is composed by a constant human and variable murine regions. In principal, the TNF-α blockers are approved for use in humans with moderate to severely active rheumatoid arthritis (RA). Among them, Etanercept seems to have the best characterized pharmacokinetic properties [Nestorov I, 2004]. Compared with ordinary methotrexate, etanercept is more effective and better tolerated [Chen YF, 2006].

Recent studies have demonstrated that anti-TNF-α therapies (e.g. etanercept or infliximab) improve inflammation-related endothelial dysfunction in various pathophysiological conditions, such as rheumatoid arthritis [Hürlimann D, 2002], heart failure [Fichtlscherer S, 2001], aging [Csiszar A, 2007], and autoimmune sensorineural hearing loss [Staecker H, 2002; Wang X, 2003; Cohen S, 2005; Van Wijk F, 2006; Street I, 2006; Lobo D, 2006]. TNF-α blockers have shown their protective cardiovascular effects against oxidative stress by a mechanism upregulating NAD(P)H oxidase and inducing nitric oxide synthase [Ungvari Z, 2003]. In inflammed middle ear mucosa, TNF-α antagonists can reduce capillary permeability, subepithelial edema, and inflammatory cell infiltration [Kim DH, 2006]. Despite the association of loud noise and the increased expression of TNF-

α, there has never been any study that investigated vascular effects of TNF-α inhibitors in NIHL.

Additionally, it is also proposed that cochlear degeneration due to immune cell infiltration can lead to permanent hearing loss [Ma C, 2000; Satoh H, 2002]. Hence, we suggest that the use of TNF-α blockers, such as etanercept, might be able to restore cochlear function and prevent permanent threshold shift by maintaining cochlear blood flow and suppressing inflammatory- responses.

3. Hypothesis and objectives

Based on a comprehensive literature review, we hypothesized that (1) loud noise can cause hearing loss by impaired cochlear blood flow. On the other hand, in order to prevent hearing loss, homeostasis and adequate oxygenation of cochlear tissues must be maintained by adequate cochlear microcirculation. Since TNF-α is important for SMA vasoconstriction, we also hypothesized that (2) the administration of a TNF-α inhibitor (such as etanercept) after noise exposure can improve cochlear blood flow and reduce NIHL.

Objectives of the study were to test our hypotheses and to:

(1) Establish a new acute NIHL model for *in vivo* analysis of cochlear microcirculation and hearing function in guinea pigs,
(2) Confirm the effects of loud noise on cochlear microcirculation and hearing function,
(3) Evaluate the effects of etanercept on cochlear microcirculation and hearing function after acute NIHL**,** and find out whether etanercept can improve cochlear blood flow and reduce hearing loss.

II MATERIALS AND METHODS

The protocols were performed in accordance with the policy on the use of animals, as endorsed by the National Research Council of the USA and fulfilled the requirements of German law. Both two parts of the study were conducted in the Walter-Brendel-Center for Experimental Medicine (WBex) during October 2007 to September 2008.

Experiment part I

1. Establishment of a new model for *in vivo* analysis of cochlear microcirculation and hearing function of the inner ear after loud noise exposure

1.1 Animals

Male albino Hartley guinea pigs, weighing 250 to 400 g were used in this study. The animals were equally divided into 2 groups, a noise-exposed and a control (unexposed) group (n=6 per group). Animals in both groups underwent identical surgical preparations, however only one group was exposed to loud noise for 30 min.

Before study began, guinea pigs were determined whether they were sound responsive or deaf by performing a Preyer's reflex test. When a brief movement of the animal's pinna (contraction of postauricular muscle towards the cranium) or the rapid movement of the whole body was observed in response to a 30-cm-distance handclap, the animal was classified as having a normal Preyer's reflex.

All guinea pigs with a normal Preyer's reflex were initially sedated by gas anesthesia (a mixture of 1.5% Halothane in 1.5 L/min Oxygen plus 2 L/min Nitrous oxide) for approximately 5 min. Then, the halothane was turned off and the animals were purely anesthetized with intraperitoneal injection of a combination of Ketamine 85 mg/kg (Ketavet®; Parke-Davis, Berlin, Germany) and Xylazine 8.5 mg/kg (Rompun®; Bayer, Leverkusen, Germany). A surgical level of anesthesia was maintained by supplementary Ketamine and Xylazine every 45 min (~ half-dose). This anesthetic protocol

was proven very reliable for maintaining systemic blood pressure. As noted, Halothane and Nitrous oxide gas was instantly turned off after the intraperitoneal anesthesia had been given. Only 3 to 3.5 L/min of oxygen were delivered through an inhalation mask during the entire period.

1.2 Surgical preparation

1.2.1 Basic surgical preparation and monitoring

Under general anesthesia, an animal was shaved at 6 different areas: (1) left inguinal area including the upper thigh, (2) right anterolateral neck, (3) right postauricular area, (4) vertex area, (5) over the left mastoid area, and (6) middle of the back. Hairs were then removed and a slight amount of EMLA cream (lidocaine 2.5% + prilocaine 2.5%, AstraZeneca LP, DE, USA) was applied in order to prepare the skin for the surgical procedure.

The animal was placed in a supine position under an operating microscope. A skin incision of 2-cm size was made at the left inguinal area along the contour of left femoral artery. A gentle dissection was performed in order to separate the artery from the femoral nerve and vein. To prevent arterial vasospasm due to our surgical manipulation, we used a minimal amount of lidocaine spray over the vessels before dissection. When a distal portion of femoral artery had been ligated by Silk 4/0, a polyethylene catheter (PE 50) was inserted into the proximal femoral artery to facilitate monitoring of systemic blood pressure and obtain arterial blood sample for arterial blood gas and serum electrolyte evaluation. Mean arterial pressure (MAP) was monitored thoroughly and displayed on a PC screen nearby (*Daisy Lab* 5.0, Datalog Gmbh, Mönchengladbach, Germany).

For intravenous administration, the right external jugular vein was exposed. It was cannulated with another microcatheter filled with 0.9% saline solution (NSS). During the experiment, animals were placed on a thermostatically controlled heating pad. Their core body temperature was maintained at 38 $\pm$ 1 °C. Heart rate and oxygen saturation were monitored thoroughly by pulse oxymetry. Since the animals were allowed to breathe spontaneously, a careful observation of adequate airway and a supplementary oxygenation was provided throughout the experiment.

1.2.2 Surgical preparation of the ear

Under a microscope, a postauricular approach was performed on the right ear with the animal in prone position - its head slightly turned to the right

side. (Figure 8, 9) Hairs had been shaved and removed before the first incision was made.

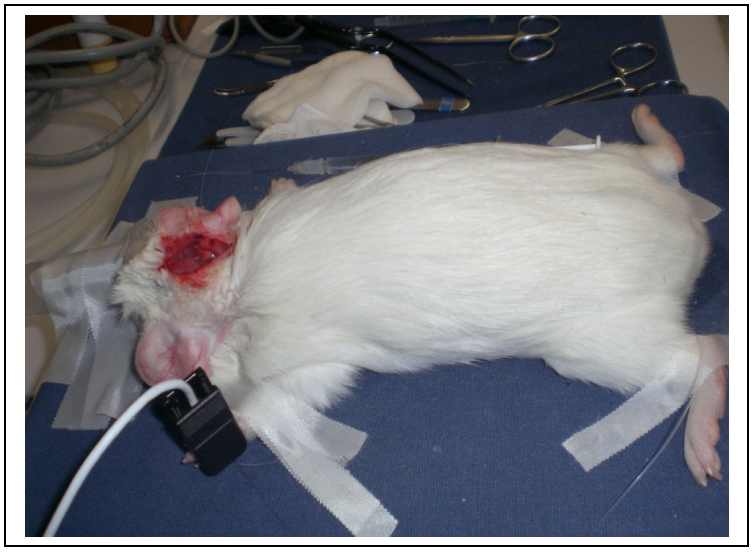

Figure 8 *A guinea pig placed in a prone position with head turned to the right side.*

Figure 9 Postauricular incision created on the right ear.

Skin, subcutaneous tissue, and fascia were divided layer by layer. A hanging suture was made by 3/0 silk so that the right pinna could be retracted antero-laterally. Temporalis muscle was then dissected away from the skull. Also the periosteum had been removed (Figure 10). Bleeding that

occurred during surgery was stopped by electrocauterization and pressure-dressing techniques. Right mastoid bulla was identified and removed carefully. Normal structures in a middle ear cavity were exposed and inspected. The tympanic membrane, annulus, facial nerve, and ear ossicles were identified. Posterior annulus, the posterosuperior part of tympanic membrane and all ear ossicles were then sacrificed with the intention to provide a better access to the cochlea (Figure 11).

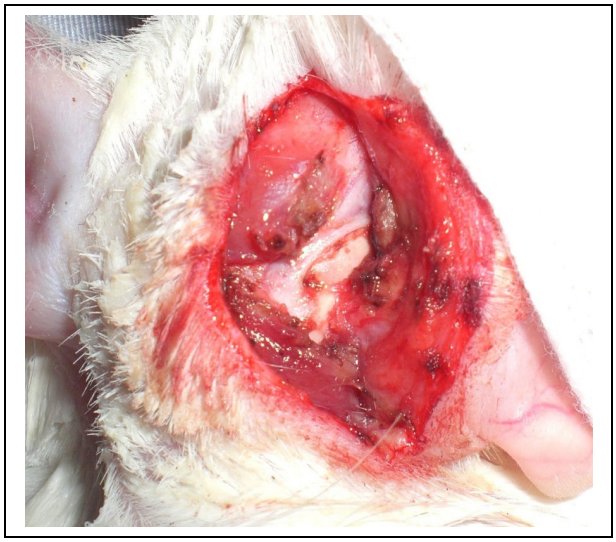

Figure 10 *Temporalis muscle cut to periosteum.*

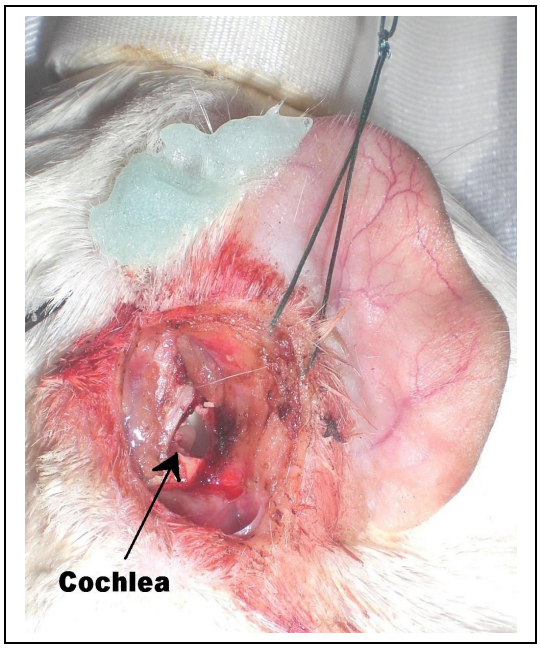

Figure 11 *The mastoid bulla and ossicular chain were gently removed. The cochlea (black arrow) was seen underneath.*

1.2.3 Cochlear window

The mucosa overlying the second and the third turn of the cochlea were gently wiped off by a piece of gelfoam. A small rectangular window (0.2 x 0.3 mm in size) was meticulously created over the convex part of the second turn by using a small knife blade (No. 11 scalpel), elevating the cochlear bony wall without traumatization to the underlying spiral ligament and stria vascularis. Only a tiny and intact piece of thin bone of the cochlear lateral wall was removed from the entire cochlea (Figure 12). The spiral ligament and strial vessels were then clearly exposed within the created window.

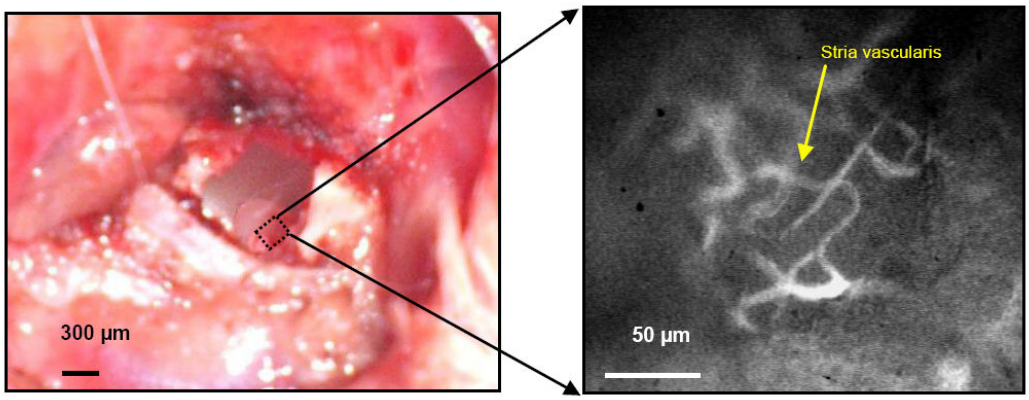

Figure 12 *(left) A small rectangular window was created and a thin bone was subsequently removed from the entire cochlea (right). Capillaries in stria vascularis were seen under a fluorescence microscope after intravenous injection of a fluorescent dye (FITC-dextran).*

1.3 In vivo fluorescence microscopy of the inner ear

After the surgical procedure animals were transferred from the operating table to a modified Zeiss microscope (Axiotech Vario; Zeiss, Goettingen, Germany). They were laid in prone position on a thermoregulated heating pad. Supplement oxygenation was provided as usual. After Fluorescein isothiocyanate (FITC)-labelled dextran was injected into the jugular vein; regions of interest within the cochlear window were focused under a 2.5x objective (Zeiss Plan-NEOFLUAR, Göttingen, Germany). Higher magnification of the respective area of strial capillaries was obtained from a long distance 20x objective (Olympus SLMPlan, Hamburg, Germany).

1.3.1 Fluorescein isothiocyanato dextran (FITC)-labeled dextran as a plasma marker

FITC-labeled dextran is a suitable fluorescent marker for analysis of the microvascular rheology to tissues. FITC conjugated to dextran was injected intravenously as a plasma marker to visualize cochlear microcirculation prior to IVM. FITC is supplied as a yellow powder which freely dissolves in water, thereby giving a bright yellow solution. Special dextran fractions prepared from native Dextran B512F (Sigma Chemical Company, St. Louis, USA) were labeled with fluorescein using the technique described by de Belder and Granath [de Belder AN, 1973]. Fluorescein particles are attached by a stable thiocarbamoyl linkage without any depolymerization of the dextran during the labeling process. It has also been confirmed that the permeability properties of FITC-labeled dextran are fundamentally unchanged [Hulström D, 1983].

FITC-substituents are stable at normal pH environment at room temperature. They distribute evenly over the dextran molecules. It has been shown in several studies that FITC conjugated to dextran is stable in vivo and has an excellent biocompatibility profile.

In order to prepare the solution, sterilization by a sterile filtration must be performed. FITC-dextrans are basically available at various molecular weights (MW), depending on permeability properties of the microvasculature of interest [Olsson Y, 1975; Hulström D, 1983]. For example, a FITC-labeled dextran at MW 150.000 will be retained in the blood vessels since the fraction excreted by the kidneys is very low.

For cochlear microcirculatory evaluation, we used FITC-labeled dextran at MW 500.000 (0.05-0.1 mL of a 5% solution in 9% NaCl; Sigma, Deisenhofen, Germany). A dose of 15 mg/100 g (50 mg/mL in physiological saline) was infused slowly over a 5-min period.

1.3.2 IVM

In vivo fluorescence microscopy was already set and ready to use. The experimental setup provided a connection from the microscope to video-documentary unit and a monitor screen for subsequent off-line analysis. The use of IVM as a tool for studying the microcirculation has been employed for several decades. Up to present, the associated techniques and tools have

been well-developed, including the software for on-line data processing. For example, Harris et al. (Munich, Germany) [Harris AG, 1997] built a more ergodynamic microscope to minimize light exposure to the tissues. The cameras for fluorescence imaging, such as silicon-intensified-target (SIT) camera, have been created to offer clearer images with less multiplication fluctuation than original tube camera and some charge-coupled device (CCD) cameras.[Amos WB, 1999; Ohshima N, 2006]. Klyscz et al. [T. Klyscz, 1997] provided computer-assisted video image analysis systems that allowed several integrated analysis functions, including red blood cell velocitiy measurements using the line shift diagram method, the spatial correlation method or the auto flying spot method.

In this study, selective observation of FITC-labeled plasma was carried out using the similar setting as Strieth et al. have described [Strieth S, 2004; Strieth S, 2006; Strieth S, 2008]. This intravital microscopic unit consists of:

- a laboratory microscope (Axiotech vario; Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with precision lifting stage
- an electrical object stage (controllable in X-Y direction) with integrated fiber-optic transmitted light (Fa. Märzhäuser, Wetzlar, Germany)
- Objective lens (SLMPlan x20; Olympus, Hamburg, Germany)
- a 100-W mercury lamp (HBO 103 W/2; Zeiss, Göttingen, Germany)
- a Ploemopack illuminator with a Leitz I2/3 filter block (excitation 450-490 nm, emission ≥ 515 nm) (Zeiss, Göttingen, Germany)
- a SIT video camera (C2400-08; Hamamatsu, Herrsching, Germany)
- a monitor screen (Television; Sony, Tokyo, Japan)
- a video timer (VTG 33; For-A, Tokyo, Japan)
- a digital videocassette recorder (DSR-45P; Sony, Tokyo, Japan)
- Digital videocassettes (AY-DV124AMQ; Panasonic, NJ, USA)

Epi-illumination was achieved by a mercury lamp with specific fluorescence filters. Excitation wavelength for FITC was 490 nm and fluorescence intensity was measured at 520 nm. Images of strial capillaries were acquired using a SIT camera, accompanied by real-time monitoring and recorded on digital videocassettes (DV) by DV recorder for subsequent off-line analysis.

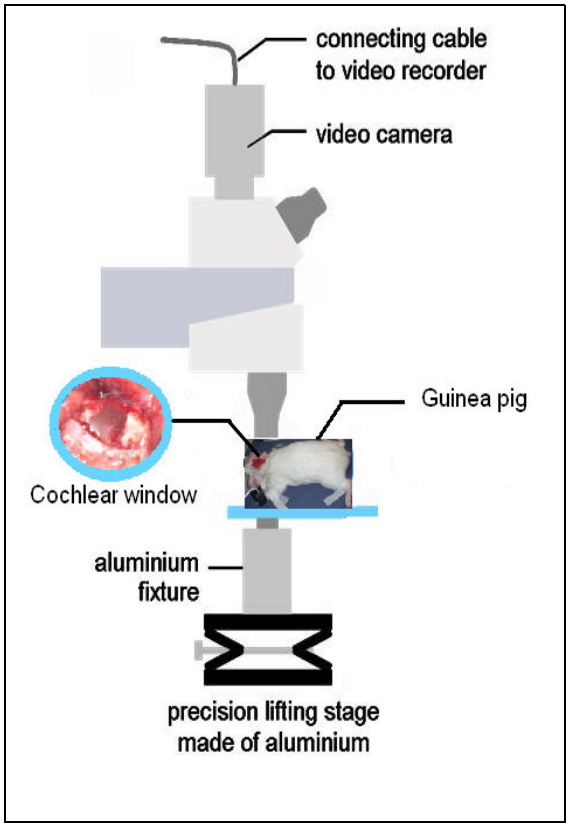

Figure 13
Picture demonstrating of the experimental setup for intravital fluorescence microscopy (IVM). A video camera and a video recorder were connected.

1.3.3 Digital video record for subsequent off-line analysis

The picture of microcirculation in strial capillaries was acquired by a SIT video camera and recorded digitally for later off-line analysis (Figure 13). In this study, we used SIT video camera, which is one of the high sensitivity cameras that boost signal strength by the electron multiplication method. It is capable of capturing images at a frame rate that is appropriate for determination of capillary red blood cell velocity [Ohshima N, 2006]. Blood flow was recorded soon after the cochlear window had been opened. Therefore, initial red blood cell velocities (v_{RBC}) in each animal were designed to be collected from the 60^{th} min, meant 30 min of noise exposure plus 30 min of operation in noise-exposed animals or 30 min without manipulations plus 30 min of operation in the controls, and repeatedly recorded every next 30 min up to *210 min*. (see Figure 15)

1.4 Microcirculatory analysis

Analysis of microcirculatory parameters was performed by an image analysis system (Cap Image; Zeintl, Heidelberg, Germany). This system

was described in detail by Zeintl et al. [Zeintl H, 1989] and Klyscz et al. [Klyscz T, 1997] and allowed measurement of functional vessel density (fvd) as a parameter of angiogenic activity. Red blood cell velocities in selected vessels (v_{RBC}) were other primary values obtained from the analysis system. With regard to the the vessel diameters (d), the blood flow in vessel segments (Q) was calculated according to the equation first described by Baker and Wayland [Baker M, 1974]:

$$Q = \frac{v_{RBC}}{1.6} \bullet \left(\frac{d}{2} \right)^2 \bullet \pi$$

1.5 Evaluation of hearing function

Auditory function was assessed by recording ABR. Each animal was anesthetized with Ketamine 85 mg/kg plus Xylazine 8.5 mg/kg i.p., and kept warm on a thermostatic-controlled heating box as usual. Evaluation of hearing function was performed in both ears for the first time during the beginning of the experiment, labeled as timepoint *0 min*. Afterwards, only thresholds on the left ear were recorded repeatedly every 30 min up to *180 min*.

1.5.1 Evoked auditory brainstem responses (ABR)

Response to a sound by ear, auditory nerve, and lower brain can be tested with ABR. The ABR test was performed after attachment of the electrodes to the head in order to record electrical activity from the auditory nerve and lower brain. Generally, ABR test can be used for hearing threshold evaluation in infants or young children who are not eligible for standard audiometry, or to distinguish between a cochlear and a retrocochlear lesion.

1.5.2 Preparation for ABR measurement

Generation of acoustic stimuli and subsequent recording of evoked potentials were performed using a GSI Audera® (VIASYS HealthCare Inc., Wisconsin, USA). The measurements of ABR thresholds were recorded at 8 kHz. Needle electrodes were placed subcutaneously over a vertex area

(ground) for positive recordings, at an area ventrolateral to the pinna or over the mastoid tip of the measured ear for negative recordings (active), and at the midline area of the back as the reference (Figure 14). The electrodes were connected to the measuring instrument via an electrode connector box for integrated impedance measurement. The data acquisition system regulated the stimulus-response. Amplification was controlled by the measuring program, the signal filter and analog-digital conversion.

Prior to ABR measurements, all animals were examined under a microscope to have normal tympanic membranes. Both external ear canals were cleaned to be free from debris. The impedance was also checked by using the impedance testing features provided in the GSI Audera® amplifier until at least 5 kΩ resistance values were achieved. During ABR measurements, it is advisable to turn off the thermoregulated heating box to avoid external electrical induction and potentials which may interfere with the ABR waveforms.

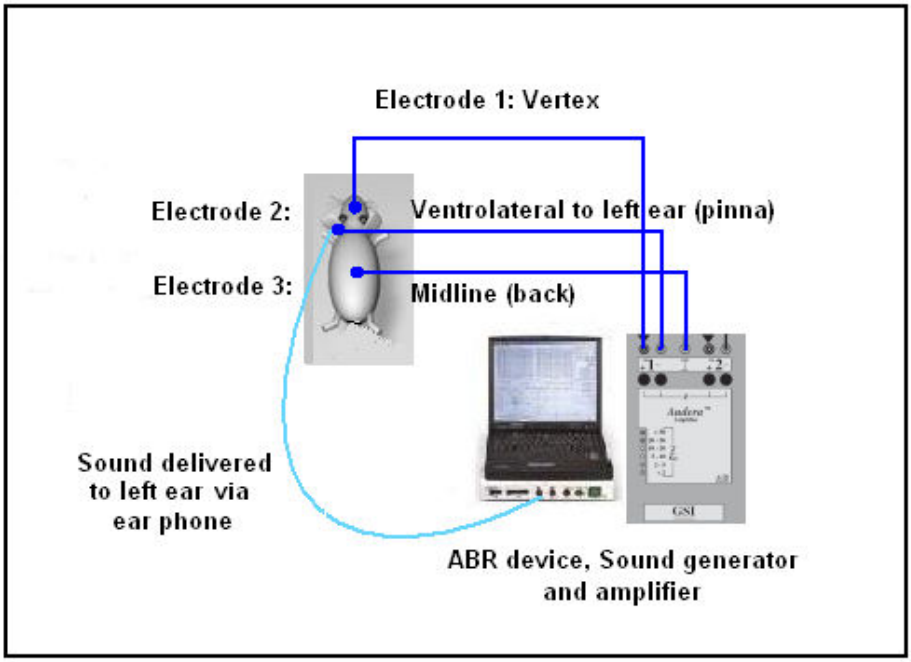

Figure 14 *Subcutaneous ABR electrodes were placed at (1) the vertex area, (2) the ventrolateral area of measured ear, and (3) midline of the back. The acoustic stimuli was generated by a sound generator and delivered to the right auditory canal through a tubal insert-phone foam tip.*

1.5.3 ABR measurement and data analysis

Acoustic stimuli consisting of 100µs-tone-bursts at 8 kHz with a repetition rate of 30/s, was delivered monaurally through a tubal insert-phone foam tip fitted well to the animals' ear canals. Hearing threshold, defined as the lowest intensity of stimulation that yielded a recognisable ABR wave III and IV, was determined from a set of responses at various intensities. The supraliminal potentials were recorded in 10-dB steps, whereas the near-threshold potentials were recorded in 5-dB steps. Electrical signals were averaged after 1024 repetitions. Threshold at each evaluation was verified at least twice.

Initial auditory function was measured at timepoint *0 min*. Hearing thresholds at this timepoint indicated auditory function of both ears before the animals were exposed to loud noise as well as prior to right ear operation (both groups). Afterwards, only evaluation of the left ear had been performed. Serial ABR thresholds were repeated every 30 min up to 180 min (timepoint *180 min*).

1.6 Noise exposure

For 6 animals in the noise-exposed group, noise was generated by a sound generator (GSI Audera®; VIASYS HealthCare Inc., Wisconsin, US), amplified by Audera® amplifier and delivered through the ear phones. A noise (octave-band centered at 4-kHz) of 106 dB SPL was used as an exposure stimulus. Loud noise was delivered continuously for 30 min over the right and left ears respectively to induce injury to the cochleae.

1.7 Experimental protocol

During the establishment of the new animal model for *in vivo* analysis of cochlear microcirculation and hearing function after acute loud noise, animals were divided into 2 groups (See Figure 15):

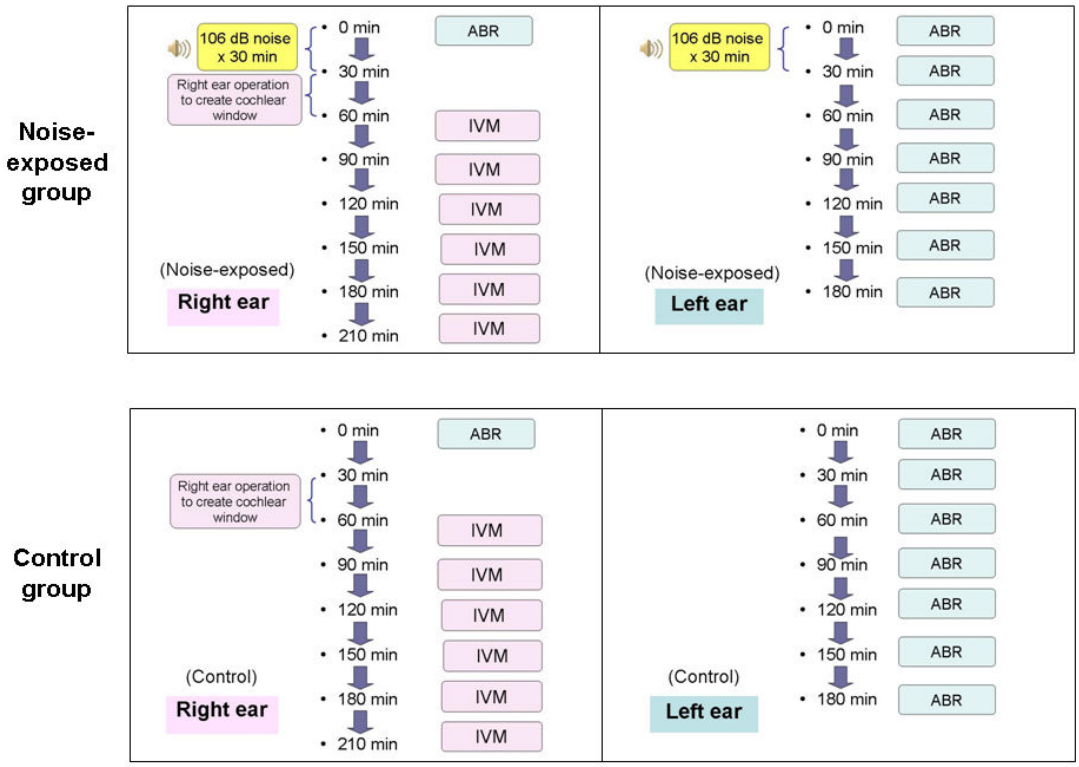

Figure 15 *Experimental protocol for the experimental part I*

1.7.1 Noise-exposed group

Before the right ear was operated, six animals had been exposed to loud noise, 106 dB SPL, for 30 min. Continuous loud noise was delivered from the same sound generator directly to animals' auditory meatus of the right and the left ear, respectively. While the right ear was surgically opened, loud noise was delivered to the left ear. After that, animals had been moved to the fluorescence microscope for the first analysis of cochlear microcirculation (beginning at timepoint *60 min*). Serial cochlear microcirculatory evaluations were performed on the right ear every 30 min until timepoint *210 min*. On the left ear, evaluation of hearing thresholds began at timepoint *0 min* up to timepoint *180 min*, with repetition every 30 min.

1.7.2 Control group

The other six animals served as controls. These animals received identical surgical procedures, microcirculatory and ABR hearing threshold evaluations, but were not exposed to any loud noise during 0 to 30 min.

1.8 Statistical analysis

Statistical analysis was performed by using SigmaStat 3.1 (Systat Software, Inc., California, USA). Results are presented as mean ± SD. Values of independent groups were compared with the Kruskal-Wallis and the Mann-Whitney *U* test. The Spearman's coefficient was calculated to analyze correlation. *P* values smaller than 5% were considered to be significant.

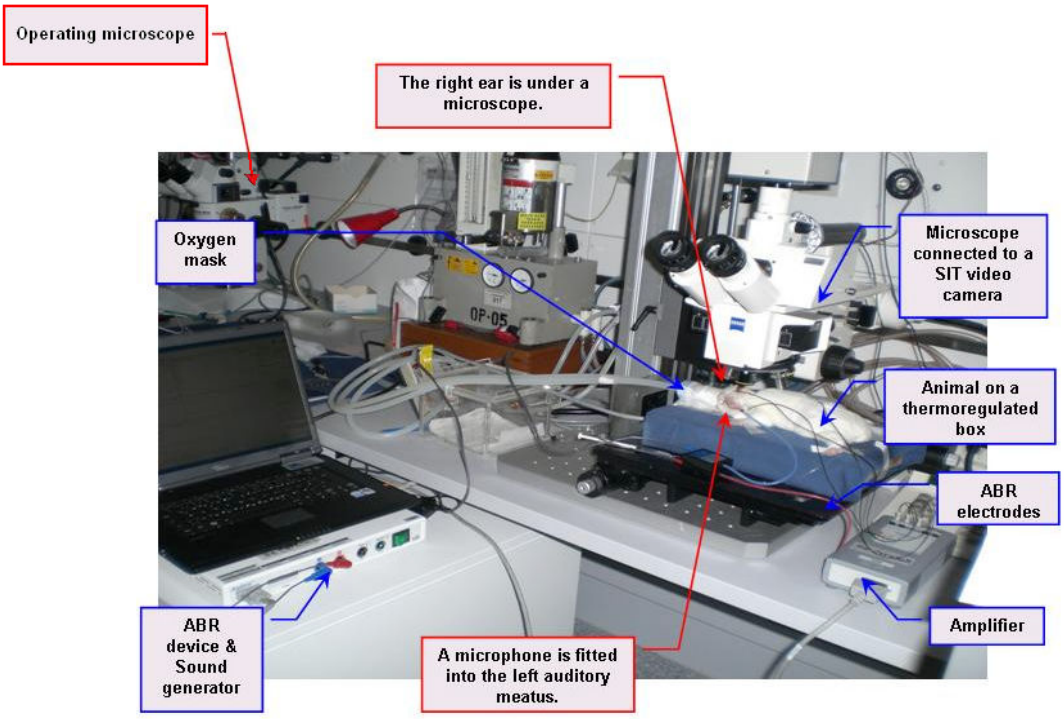

Figure 16 *Experimental setup combined IVM and ABR measurements. After the right cochlea window had been successfully prepared, the animal was moved from operating microscope (left) to the fluorescence microscope (right). Continuous oxygenation was delivered through oxygen mask accompanied by the monitoring of oxygen saturation, mean arterial pressure, and body temperature. While the images of cochlear microcirculation were recorded using a SIT video camera, hearing threshold was measured by ABR with the electrodes placed under the animals' skin and ear phone fitted into left auditory meatus.*

Experimental part II

2. Effects of a TNF-α inhibitor on cochlear microcirculation and hearing function in acute NIHL

To test the hypothesis that anti-TNF-α therapy yields vasoprotective effects in the inner ear after noise injury, the acute NIHL model (see experimental part I) was used. All animals were exposed to 106 dB broad-band noise, centered at 4 kHz, for 30 min.

2.1 Animals

Again male albino Hartley guinea pigs, weighing 250 to 400 g were used in this study. The animals were equally divided into two groups, an *etanercept*-therapy and a control group (n=6 per group). Animals in both groups experienced identical surgical preparations and the same degree of noise overstimulation. After loud noise exposure, animals in the etanercept group received systemic etanercept injection whereas the animals in the control group received normal saline solution (NSS) injection. All guinea pigs with normal Preyer's reflex were initially sedated, anesthetized, and monitored in the same manner as described in II.1.1 (experimental part I).

2.2 Surgical preparation

Similar to what described in II.1.2

2.3 In vivo fluorescence microscopy of the inner ear

Similar to II.1.3

2.4 Microcirculatory analysis

Similar to II.1.4

2.5 Evaluation of hearing function

Similar to II.1.5

2.6 Noise exposure

Noise was generated by a sound stimulator (GSI Audera®; VIASYS HealthCare Inc., Wisconsin, USA), amplified and delivered through the inserted ear phones in all animals. Broad-band noise centered at 4-kHz tone with 106 dB SPL intensity was used as an exposure stimulus. Noise was delivered continuously into right and left auditory meatus for a period of 30 min.

2.7 Experimental protocol

After exposure to loud noise, serial observation of the cochlear microcirculation of the right ear was performed every 30 min (from *60 min* up to *210 min*), while the ABR hearing threshold was monitored on the left ear (from *0 min* up to *180 min*). When the animals had been ready for the cochlear microcirculatory observation and baseline observations of vessel diameters, v_{RBC}, and segmental blood flow were recorded, 2.5 mg of TNF-α inhibitor (etanercept) (= 0.1 mL of *Enbrel*, 25 mg/mL, Wyeth-Ayerst Pharmaceutical Inc., PA, USA) dissolved in 0.2 mL NSS was injected intraperitonially to six animals. Animals treated with 0.3 mL of NSS served as controls. The post-noise injection was given only once in each animal, approximately at timepoint *75 min* refering to right ear measurements (= timepoint *45 min* of left ear measurements, respectively; see Figure 17 for schematic review of experimental protocol).

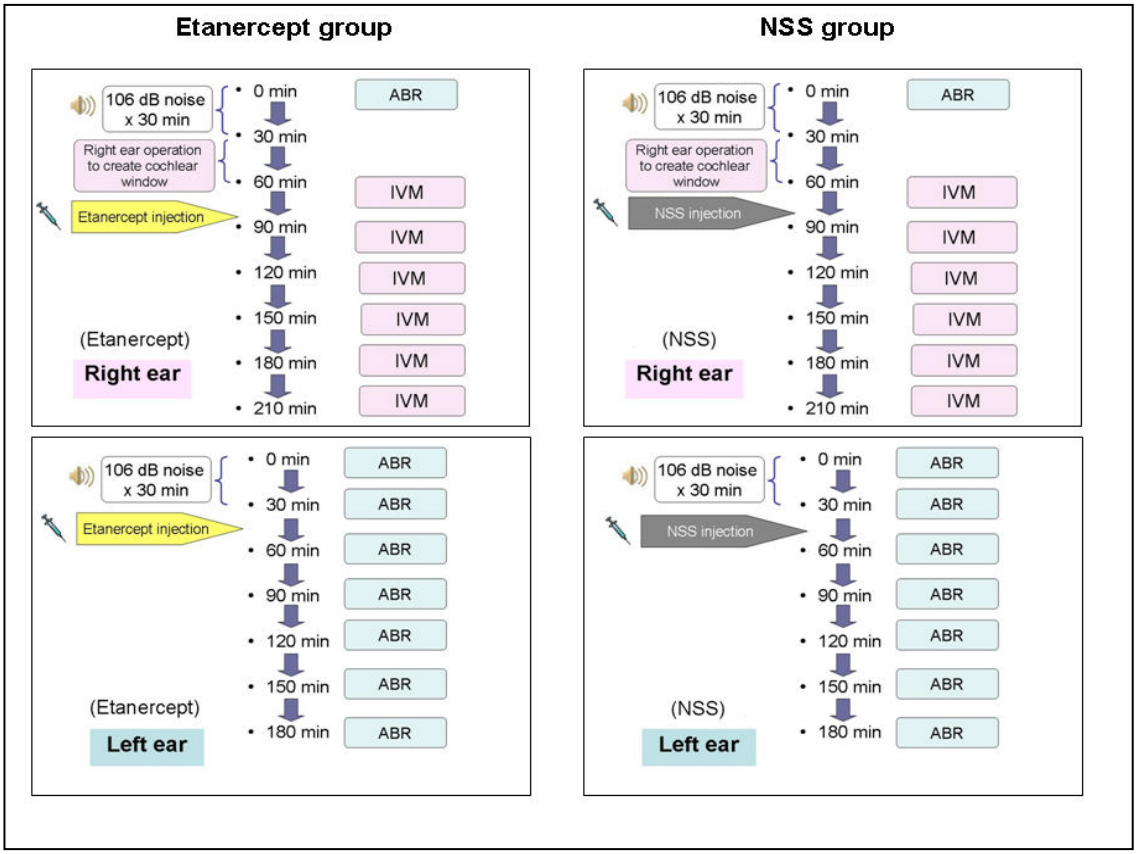

Figure 17 *Experimental protocol for the experiment part II*

2.7.1 Control group (NSS)

Animals in the control group (n=6) were treated by 0.3 mL of NSS (0.9% NaCl solution). Administration of NSS was performed by single intraperineal injection.

2.7.2 Therapy group (Etanercept)

Six animals in the therapy group were treated by a TNF-α inhibitor, etanercept. Systemic administration was performed with a single dose of 2.5 mg of etanercept (Enbrel, 25 mg/mL, Wyeth-Ayerst Pharmaceutical Inc., PA, USA) dissolved in 0.2 mL of NSS intraperitoneal injection.

TNF-α inhibitor: etanercept

Etanercept is a TNF antagonist with anti-inflammatory properties. It is a recombinant human protein consisting of the extracellular ligand-binding

domain of the human 75 kilodalton (p75) TNF-α receptor (TNFR2) linked to the Fc portion of human immunoglobulin (IgG). Etanercept (Enbrel®) was approved by the US Food and Drug Administration (FDA) for treatment of rheumatoid arthritis (RA), juvenile RA (JRA), ankylosing spondylitis (AS), and psoriatic arthritis [Mease P, 2001].

Etanercept acts as a receptor analog. It blocks TNF-α activity by competitive binding to TNF-α before TNF-α could bind to its natural receptors on the cell surface (Figure 18). With dimeric nature of soluble etanercept, the binding affinity of etanercept to molecules of TNF-α is higher than the natural monomeric forms of the TNF-α receptors [Mohler KM, 1993].

2.8 Statistical analysis

Statistical analysis was performed using SigmaStat 3.1 (Systat Software, Inc., California, USA). Results were presented as mean ± SD. Values of independent groups were compared with the Kruskal-Wallis and the Mann-Whitney *U* test. The Spearman's coefficient was calculated to analyze correlation. *P* values smaller than 5% were considered to be significant.

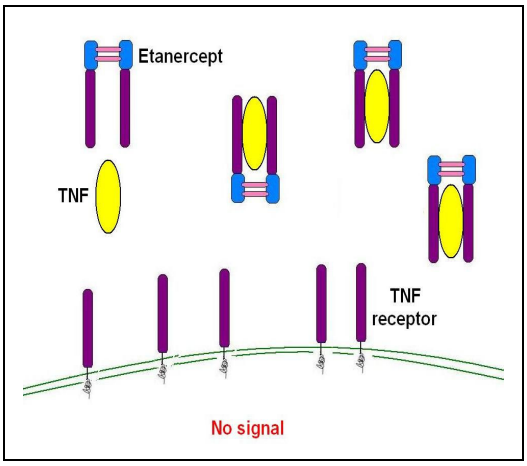

Figure 18 *The mechanism of action of etanercept is described by competitive binding to TNF-α, interrupting TNF-α/ TNF receptor.*

III RESULTS

All guinea pigs tolerated the surgical procedure well. Vital signs (body temperature, heart rates, respiratory rates, and mean arterial blood pressure were within normal ranges.

1. Cochlear microcirculation and hearing function after loud noise exposure

1.1 Cochlear microcirculation

Parameters for cochlear microcirculation measured by IVM included:

(1) Vessel diameter (d),
(2) Red blood cell velocity (v_{RBC}), and
(3) Segmental blood flow of capillaries in the stria vascularis (Q)

.

$$Q = \frac{v_{RBC}}{1.6} \bullet \left(\frac{d}{2} \right)^2 \bullet \pi$$

1.1.1 Diameters of capillaries in the stria vascularis

At the beginning of microcirculatory observation (timepoint *60 min*), the average diameter of cochlear capillaries was 9.1 ± 0.8 µm (size varied from 7.4 to 10.4 µm). Cochlear capillaries of animals in the control group revealed diameters of 9.3 ± 1.1 µm at baseline evaluation and 9.3 ± 0.7 µm at the end of the observation (timepoint *210 min*). These values were constant throughout the entire experiment. On the other hand, baseline diameters of strial vessels in noise-exposed animals were 9.4 ± 0.7 µm. At the end of the experiment vessel diameters decreased slightly to 8.8 ± 0.9 µm without statiscal significance. ($p > 0.05$) (See Figure 19)

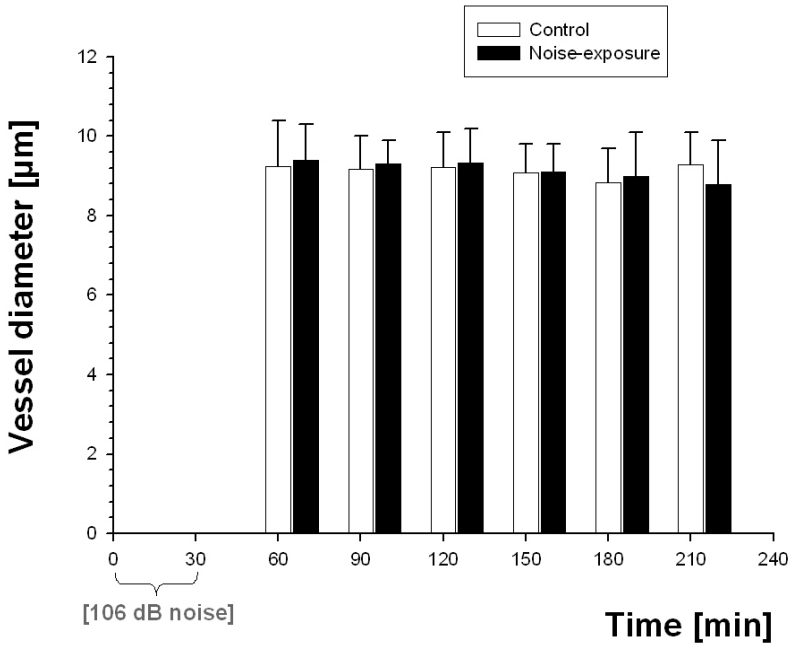

Figure 19 *Constant diameters of capillaries in the stria vascularis in noise-exposed and control animals*

1.1.2 Red blood cell velocities (v_{RBC}) in capillaries of the stria vascularis

Baseline v_{RBC} of strial capillaries from all animals, recorded at *60 min*, ranged from 102 µm/s to 144 µm/s. Average velocities were 125.3 ± 13.3 µm/s in the noise-exposure group and 124.5 ± 12.5 µm/s in the control group. Beyond baseline measurements there were statistically significant differences of v_{RBC} between the two groups starting from timepoint *120 min* ($p = 0.001$) up to the end of the observation, *210 min* ($p < 0.001$, Figure 20): a continuous decrease in v_{RBC} was observed only in the noise-exposed animals. The v_{RBC} declined from 125.3 ± 13.3 µm/s at baseline to 105.5 ± 13.4 µm/s after *90 min*. V_{RBC} were further reduced to 91.3 ± 16.4 µm/s after *120 min*, 82.7 ± 13.0 µm/s after *150 min*, and 76 ± 10.1 µm/s after *180 min*. The v_{RBC} was only 66.8 ± 11.2 µm/s at the end of the observation (*210 min*). Reductions were displayed also as percentage changes related to baseline values. The average reductions were -12.2 ± 1.3% at *90 min*, -24.3 ± 5.8% at *120 min*, -30.5 ± 5.2% at *150 min*, -36.7 ± 3.5% at *180 min*, at -44.5 ± 3.4% at *210 min*, respectively ($p < 0.001$, at every time of measurements beyond *90 min*). In contrast, within the control group, no significant change of v_{RBC} was observed during the observation period. Mean v_{RBC} ± S.D. in control animals was 118.2 ± 10.3 µm/s at *90 min* ($p = 0.262$), 121.5 ±11.6 µm/s at *120 min* ($p = 0.272$), 118.8 ± 10.0 µm/s at *150 min* ($p = 0.305$),

119.5 ± 11.9 µm/s at *180 min* (p = 0.357), and 119.7 ± 13.2 µm/s at *210 min* (p = 0.184).

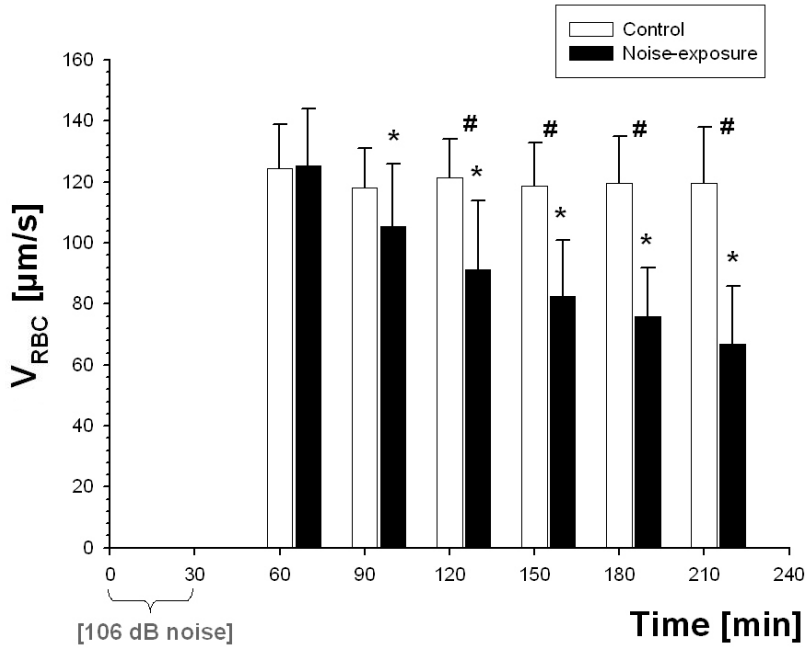

Figure 20 *Red blood cell velocities (v_{RBC}) in the strial capillaries decreased significantly after 60 min in the noise-exposure group.*

** $p < 0.05$ vs. measurements at 60 min*
 # $p < 0.05$ vs. control

1.1.3 Segmental blood flow in capillaries of the stria vascularis

Segmental blood flow of strial capillaries (Q) was calculated from v_{RBC} and vessel diameter values according to Baker and Wayland [Baker M, 1974]. Hence segmental blood flow decreased continuously after noise exposure comparable to v_{RBC}. A continuous decrease of blood flow in noise exposed animals was observed with statistical differences compared with the controls after *150 min* (Figure 21, 23). In animals exposed to loud noise, the relative changes in segmental blood flow from baseline conditions were -17.2 ± 8.2% at *90 min*, -27.7 ± 13.0% at *120 min*, -38.1 ± 8.2% at *150 min*, -44.2 ± 9.9% at *180 min*, and -53.5 ± 6.7% at *210 min*.

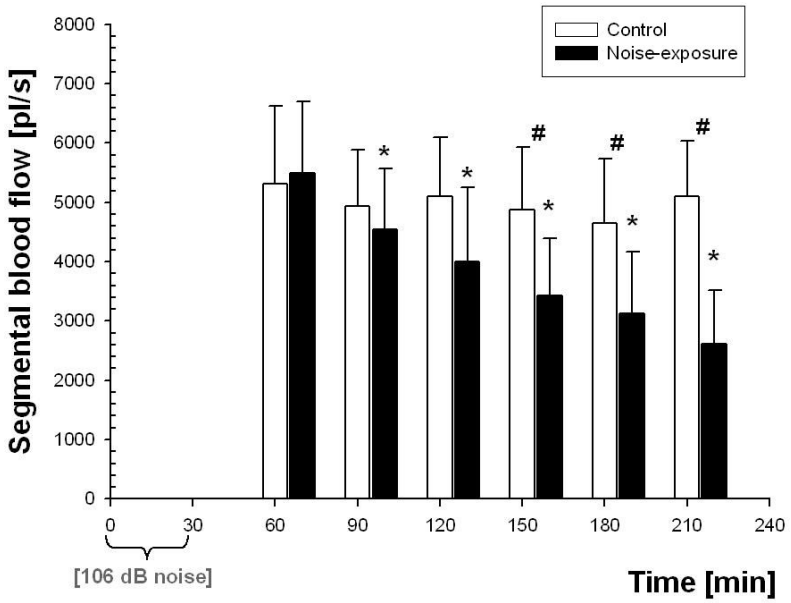

Figure 21 *Segmental blood flow of the strial capillaries decreased significantly after 60 min in the noise-exposure group.*

** $p < 0.05$ vs. measurements at 60 min*
 # $p < 0.05$ vs. control

1.2 Hearing threshold

At the beginning of the experiment, all animals presented equal hearing thresholds of both ears using ABR measurements. (Figure 22 A,B) The average hearing threshold of all animals at timepoint *0 min* was 21.7 ± 4.4 dB SPL. Baseline values were 20 ± 5.5 dB SPL in the group that was to be noise-exposed and 23.3 ± 2.6 dB SPL in the control group. After noise exposure there was a statistical difference between the noise-exposed and the control group beyond *60 min* (Figure 22 A,B) Animals after exposure to loud noise showed an average hearing threshold shift from 20 ± 5.5 dB SPL (at *0 min*) to a maximum of 45 ± 4.5 dB SPL ($p = 0.002$) at the end of the observation (*180 min*). Interestingly, hearing threshold began to rise immediately after 30 min of loud noise exposure. The subsequent threshold shifts were found to be increasing continuously. The mean hearing threshold ± S.D. was 26.7 ± 4.1 dB SPL after *30 min* ($p = 0.093$), 32.5 ± 3.8 dB SPL after *60 min* ($p = 0.002$), 34.2 ± 3.8 dB SPL after *90 min* ($p = 0.002$), 38.3 ± 4.1 dB SPL after *120 min* ($p = 0.002$), and 43.3 ± 6.1 dB SPL

after *150 min* (p = 0.002). In contrast, no change of the hearing threshold in the control group was observed (Figure 23, 24).

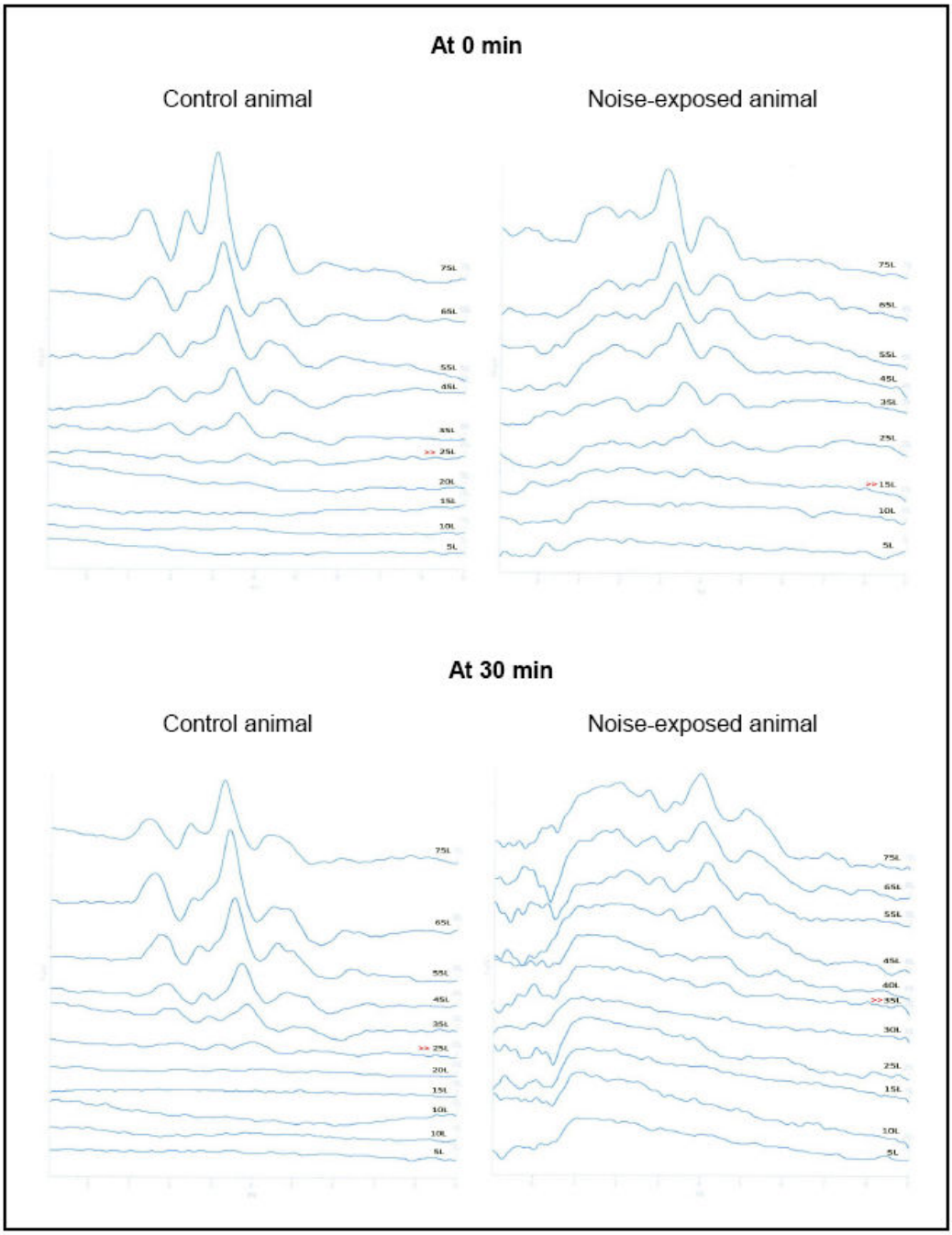

Figure 22A *Samples of ABR waves from one animal of the control group (left) and another animal of the noise-exposed group (right). Representative ABR waves at 0 min (before loud noise) and 30 min (immediate post noise) were compared to the waves of the control animal.*

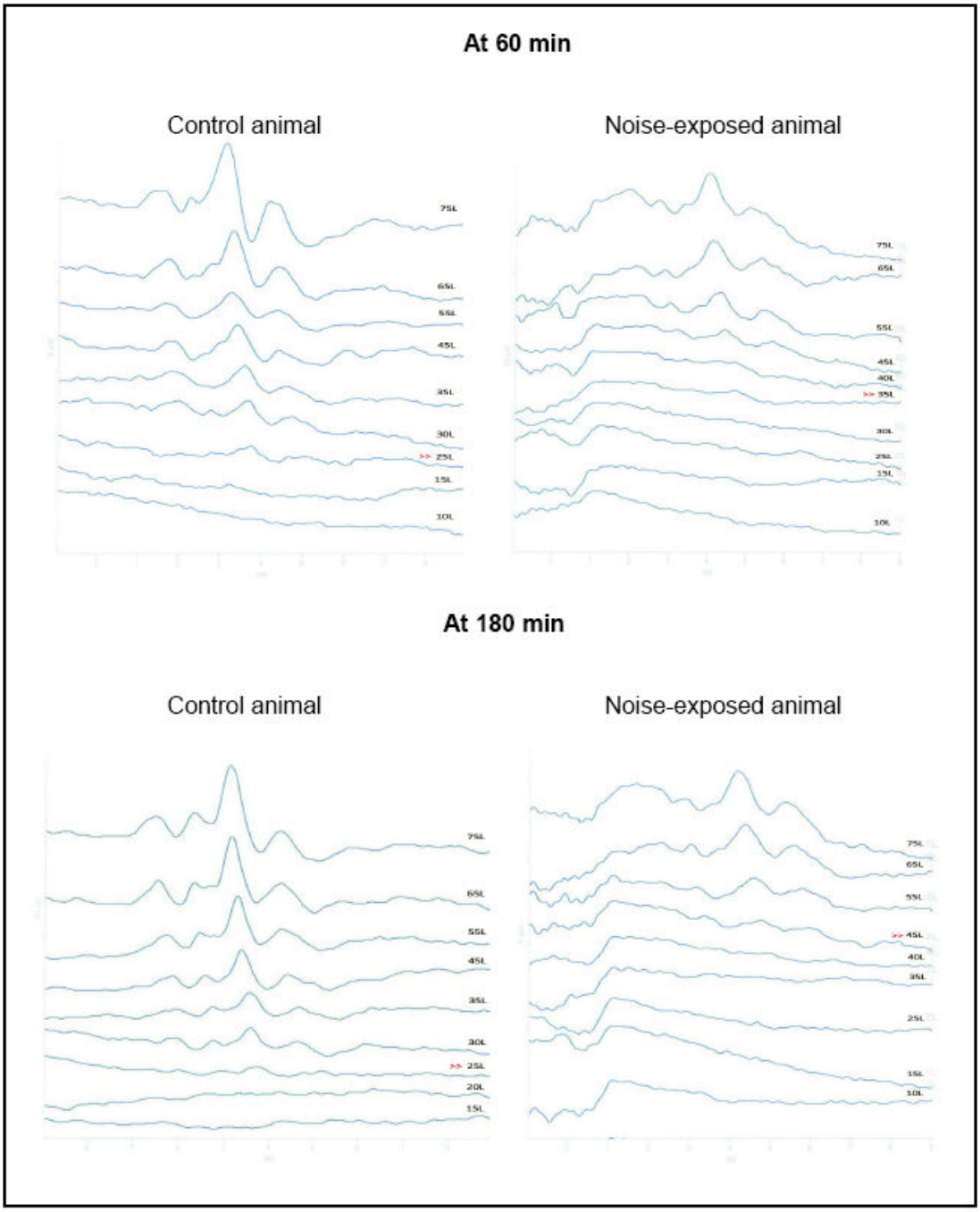

Figure 22B *Representative ABR at 60 and 180 min from the same animals with distinct ABR thresholds*: *Hearing thresholds shifted from 35 dB SPL (at 60 min) to 45 dB SPL (at 180 min) in the noise exposed animal but remained unchanged in the control.*

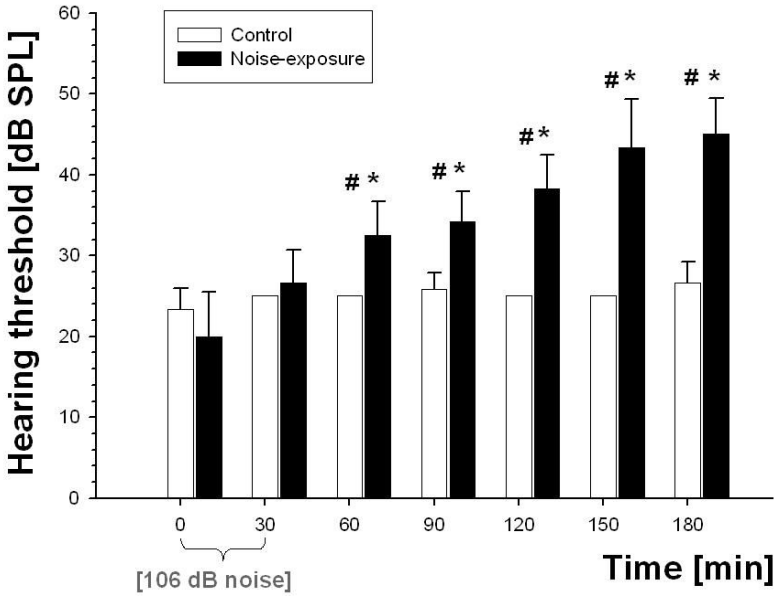

Figure 23 *Hearing thresholds increased in the noise-exposed group.*

** $p < 0.05$ vs. measurements at 0 min*
 # $p < 0.05$ vs. control

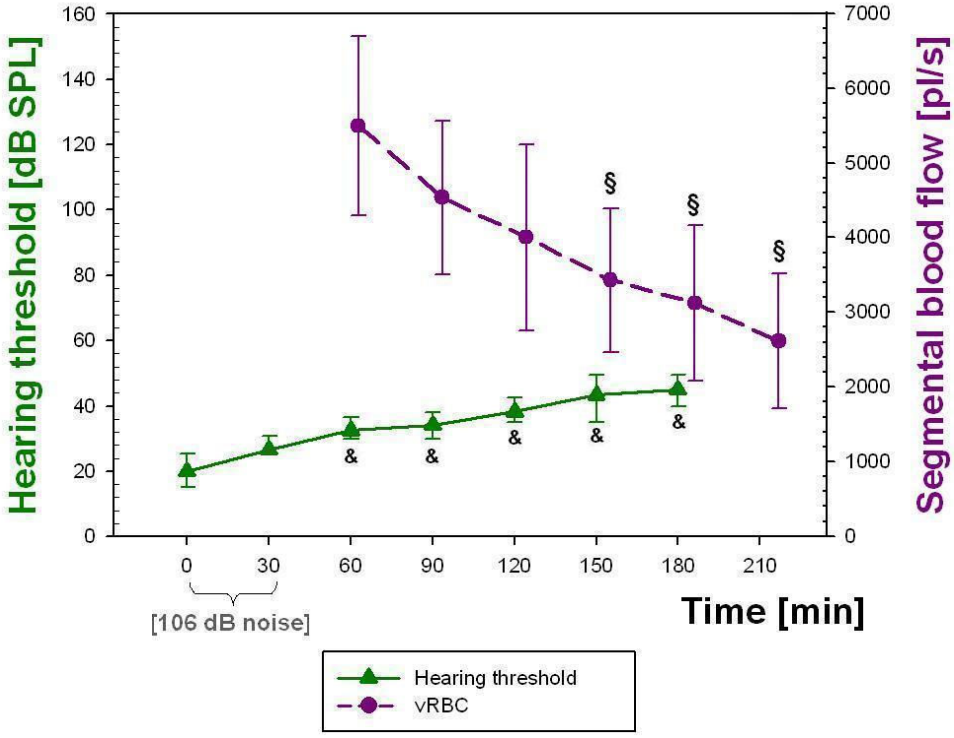

Figure 24 *Synopsis of hearing thresholds and inner ear microcirculation: after noise exposure, hearing thresholds increased as segmental blood flow decreased significantly.*

§ $p < 0.05$ vs. measurements at 60 min
& $p < 0.05$ vs. measurements at 0 min

2. Cochlear microcirculation and hearing function after TNF-α blocking treatment in acute NIHL

After the animals had been exposed to 106 dB noise for 30 min, 2.5 mg of etanercept (= 0.1 mL of Enbrel; Wyeth-Ayerst Pharmaceutical Inc., PA, USA) dissolved in 0.2 mL NSS was injected intraperitonially to six animals to test whether the TNF-α blocking therapy yielded protective effect on the microcirculation in acute NIHL. In control animals, a similar experiment protocol was conducted, however without injection of Etanercept but with 0.3mL of NSS injection instead.

2.1 Cochlear microcirculation

2.1.1 Diameters of capillaries in the stria vascularis

Average diameter of all measured vessels was 10.6 ± 0.6 μm (size varied from 9.3 to 11.4 μm). These baseline values showed no significant difference between groups ($p > 0.05$). Again there was no significant change at *60 min* and the end of experiment (timepoint *210 min*) in both groups (Figure 25). At timepoint *210 min*, mean diameter ± S.D. of vessels from animals treated with etanercept was 10.6 ± 0.9 μm, and the mean values at the final measurement from animals treating with NSS was 10.2 ± 0.9 μm**.**

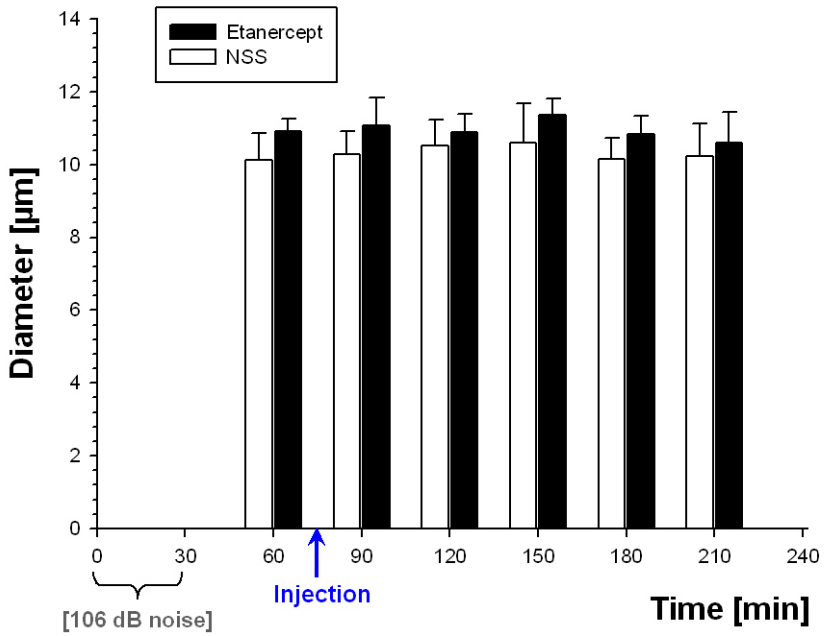

Figure 25 *Unchanged diameters of strial capillaries in animals under TNF-α blocking therapy and controls.*

2.1.2 Red blood cell velocities (v_{RBC}) in capillaries of the stria vascularis

Initial v_{RBC} in strial capillaries, recorded at *60 min*, from animals in the anti-TNF-α-therapy group was 146.8 ± 24.3 µm/s; while the initial v_{RBC} of animals in the NSS- group was 140.7 ± 27.2 µm/s. There was no significant difference between the baseline values of these two groups ($p = 0.589$). After animals had been treated with etanercept, they showed a slight increase of v_{RBC} at timepoint *90 min* with a mean v_{RBC} of 168.7 ± 27.0 µm/s. This was a significant microcirculatory protection compared to the 129.0 ± 30.9 µm/s of animals treated with NSS-alone at the same time ($p = 0.039$). Afterwards, v_{RBC} continuously decreased only in the group of animals that received NSS but not in the group that was treated with etanercept (Figure 26). V_{RBC} in the etanercept-therapy group was higher in every respective animal compared to animals in the control group. Single injections of etanercept rapidly raised v_{RBC} in cochlear capillaries by +15 ± 2.9 % at timepoint *90 min*, +10.1 ± 3.3 % at *120 min*, +8.0 ± 4.5 % at *150 min*, +6.2± 3.5 % at *180 min*, and +3.6 ± 4.1 % at *210 min* compared to baseline values. In contrast, injections of NSS alone had no effect on V_{RBC} in controls. Total reductions were -8.9 ± 5.3% at *90 min*, -16.0 ± 6.4 % at *120 min*, -23.5 ± 11.6 % at *150 min*, -27.7 ± 12.3 % at *180 min*, at -35.5 ± 12.1 % at *210 min*, respectively.

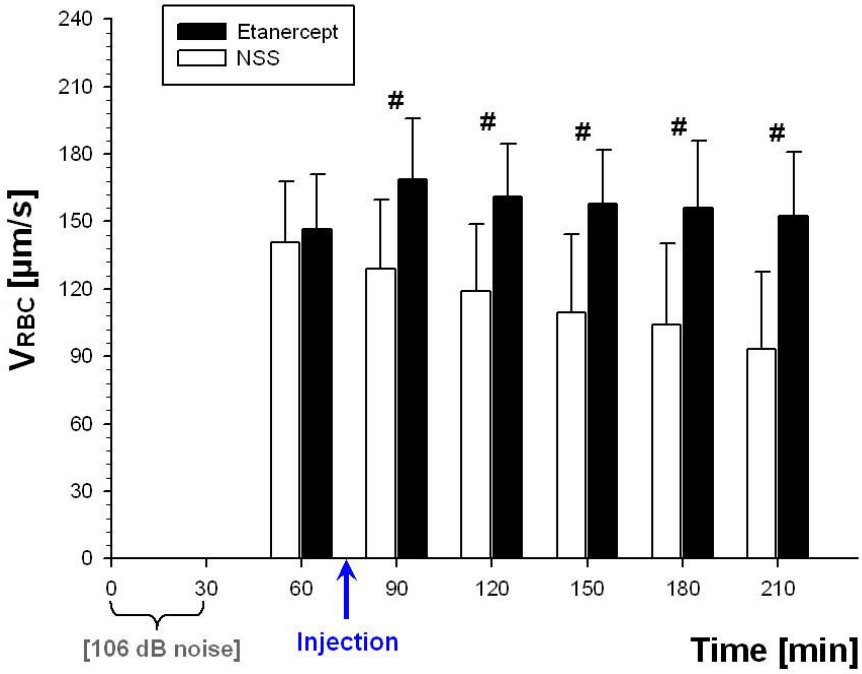

Figure 26 *After etanercept injection, v_{RBC} remained protected comparing with NSS-controls. While v_{RBC} in the control animals continuously decreased, v_{RBC} of etanercept-treated animals maintained within baseline values.*

 # $p < 0.05$ vs. NSS

2.1.3 Segmental blood flow in capillaries of the stria vascularis

In accordance to v_{RBC}, segmental blood flow in strial capillaries remained unchanged in the etanercept-therapy group but decreased continuously in NSS-controls. A slight increase of blood flow was observed immediately after the etanercept injection (at *timepoint 90 min*), yielding +19 % compared to baseline (at timepoint *60 min*) ($p > 0.05$). The segmental blood flow at timepoint *210 min* was slightly lower than the blood flow at timepoint *90 min*, however still remained within baseline values ($p > 0.05$). Further changes were +9.5 ± 7.5 % at *120 min*, +16.8 ± 7.6 % at *150 min*, +4.6 ± 7.5 % at *180 min*, at -2.2 ± 12.2 % at *210 min*, respectively. Noteworthy, every respective segmental blood flow value measured after etanercept administration was significantly higher compared to animals treated with NSS alone ($p < 0.05$). In controls, relative reductions were -9.0 ± 8.6 % at *90 min*, -12.5 ± 5.9 % at *120 min*, -19.2 ± 14.6 % at *150 min*, -29.7 ± 12.0 % at *180 min*, and -36.0 ± 15.2 % at *210 min*, respectively. Following loud noise, when segmental blood flow was reduced by -36.0% in control animals, blood flow was almost unchanged (-2.2%) in animals treated with etanercept (Figure 27, 30).

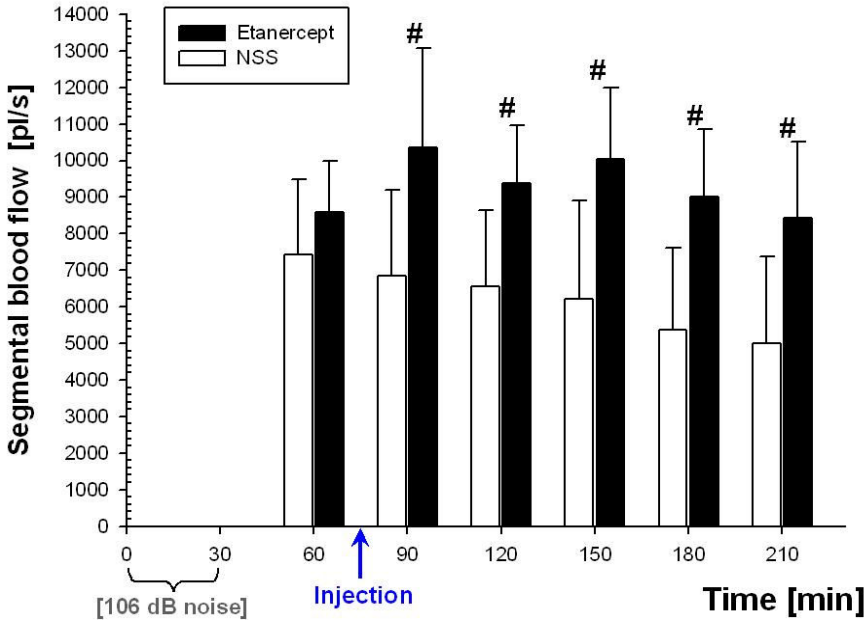

Figure 27 *Segmental blood flow in strial capillaries in the Etanercept group increased significantly compared to the NSS group.*

 # $p < 0.05$ vs. NSS

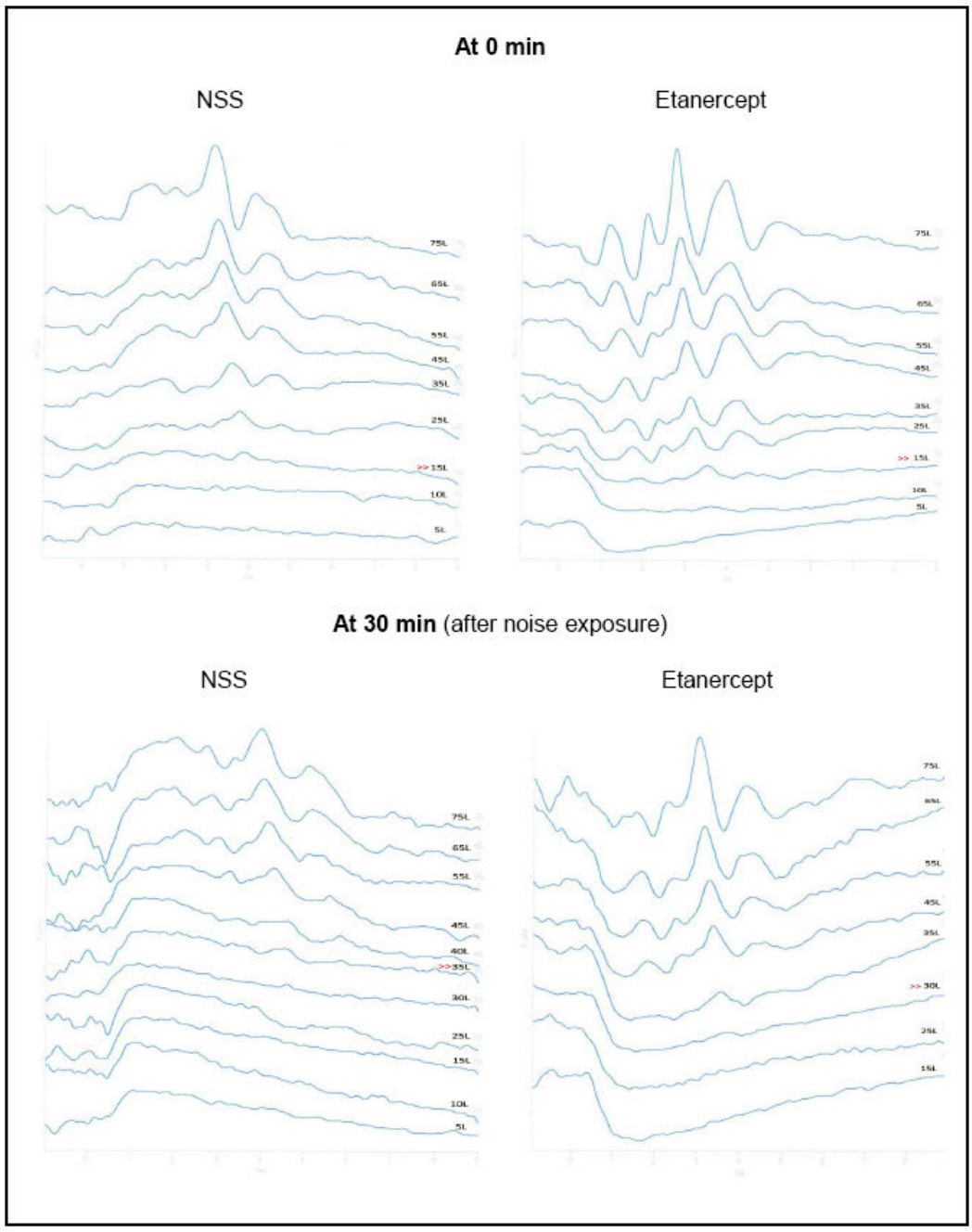

Figure 28A *Representative ABR waves at 0 and 30 min from one animal in NSS group (left) and one animal in etanercept group before treatment (right): After noise exposure, there was an immediate hearing threshold shift in both groups. Hearing thresholds shifted from 15 to 35 dB SPL in the animals of the NSS group and from 15 to 30 dB SPL in the animals of the etanercept group.*

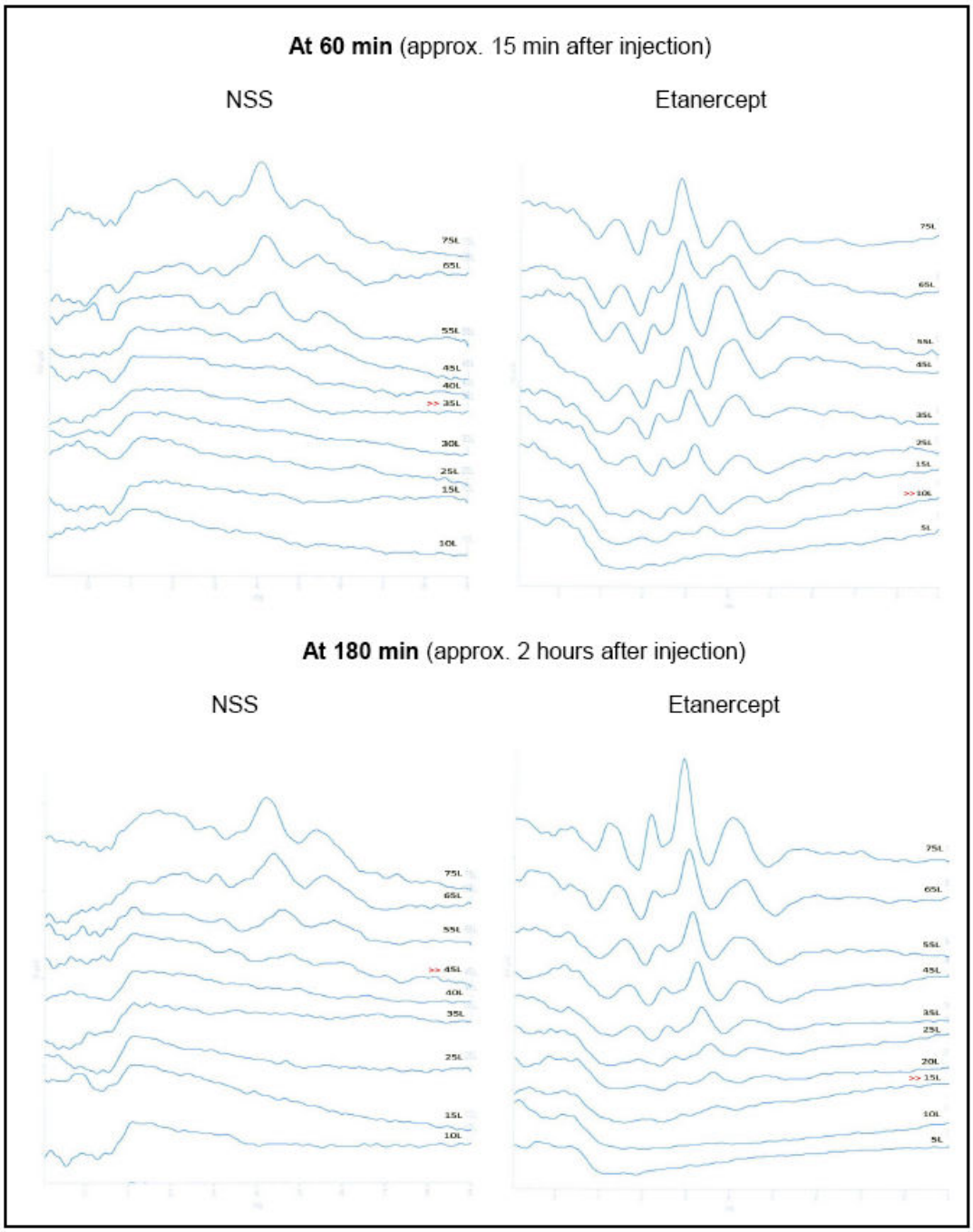

Figure 28B *Representative ABR at 60 and 180 min from the same animals after treatment with NSS (left) and etanercept (right): Hearing thereshold from the animals treated with etanercept were 10 dB SPL at timepoint 60 min and 15 dB SPL at 180 min. In contrast, hearing thresholds of the NSS-treated animals were 35 and 45 dB SPL at 60 and 180 min, respectively.*

2.2 Hearing threshold

At the beginning of the experiment, overall average hearing threshold was 20.8 ± 4.7 dB SPL among the animals. There was essentially no difference in hearing levels between the animals later treated with etanercept and the animals to be treated with NSS-alone up to timepoint *90 min* ($p = 0.015$). The maximum threshold shift in controls showed at +20 dB (at timepoint *180 min*). The average hearing threshold changed from 22.5 ± 5.2 dB SPL (at *0 min*) to 30.8 ± 3.8 dB SPL (at *30 min*) before etanercept injection (Figure 28 A,B). Similar changes ($p = 0.310$) were found in the control group, with hearing threshold changed threshold from 19.2 ± 3.8 dB SPL (at *0 min*) to 28.3 ± 2.6 dB SPL (at *30 min*) before NSS injection (Figure 28 A,B). However, after etanercept injection, the hearing threshold decreased continuously and reached its baseline levels within *180 min* (21.7 ± 2.6 dB SPL). In fact, the ABR threshold began to fall immediately after etanercept injection with the mean threshold of 26.7 ± 9.3 dB SPL after *60 min*, 25.8 ± 9.2 dB SPL after *90 min*, 24.2 ± 5.8 dB SPL after *120 min*, and 23.3 ± 4.1 dB SPL after *150 min.* In contrast, there was a continuous increase in the hearing threshold in animals treated with NSS. Maximum shift of the ABR threshold in the NSS-treated animals was +20 dB SPL, which increased from 19.2 ± 3.8 dB SPL (at *0 min*) to 39.2 ± 3.8 dB SPL (at *180 min*; Figure 29, 30).

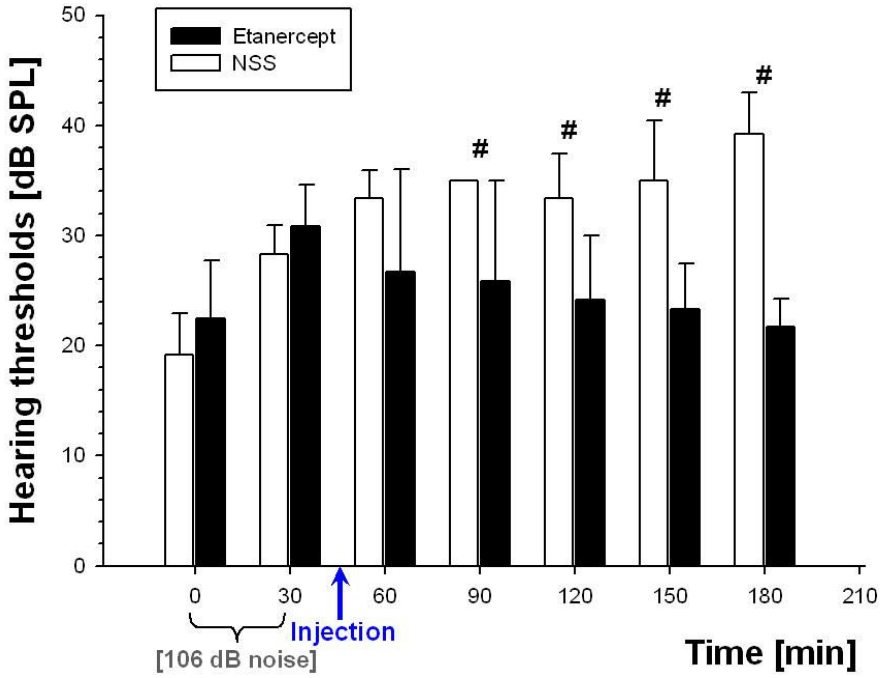

Figure 29 *Hearing threshold in the etanercept group recovered completely at the end of observation, and differed significantly from controls approximately 45 min after treatment.*

 # $p < 0.05$ vs. NSS

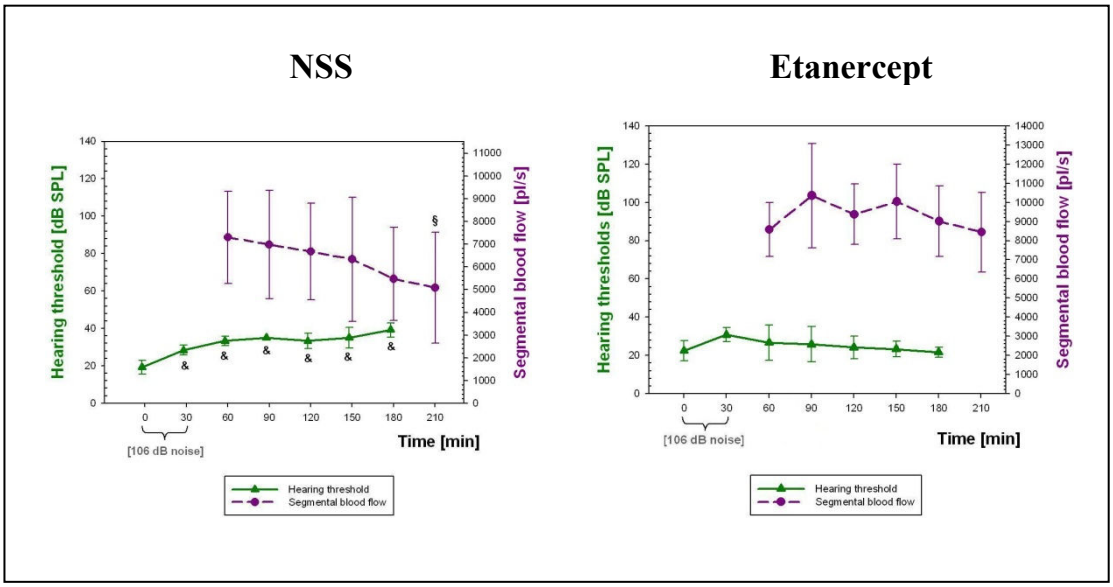

Figure 30 *(left) After loud noise, the hearing threshold gradually increased in animals receiving only NSS injection, whereas the segmental blood flow decreased. In contrast, animals receiving etanercept injections (right) presented with preserved hearing thresholds and there was no significant change of segmental blood flow from baseline.*

§ p <0.05 vs. measurements at 60 min
& p < 0.05 vs. measurements at 0 min

IV DISCUSSION

1. The model for *in vivo* analysis of cochlear microcirculation and hearing function of the inner ear after loud noise exposure

It is acknowledged that, to correct hair cell function, high potassium levels must be maintained in the endolymph fluid by active ion channels within the stria vascularis for correct hair cells function. These strial cells' blood supply is derived from capillaries, which are fed by arterioles of the SMA. Some inner ear pathologies such as vascular disorders, autoimmune diseases, as well as noise injury, can impair blood flow and thus induce sensorineural hearing loss by interference with energy demanding of endocochlear potential function. In the first part of this study, we were interested in the importance of blood flow to stria vascularis and its associated alteration following loud noise.

1.1 Methodology

This is the first study that reveals both hearing threshold shift and reduction of cochlear microcirculation after acute loud noise by using ABR and IVM. This method allows real-time *in vivo* analysis of cochlear microcirculation and hearing function by direct observation of rheological changes in cochlear capillaries and serial measurements of hearing thresholds. The experimental setup described here turned out to be higly reproducible yielding very low standard deviations of the measured values. All values obtained from control animals (including diameters, red blood cell velocities, and hearing threshold) were considerably constant with low biological variations more than 3 h of observation. The only draw back of this model was that cochlear blood flow and hearing function must be assessed from different ears. Nevertheless, both parameters were measured in the same animal and hearing threshold was verfied on both ears prior to the experiment, respectively.

Guinea pigs were used in this study because they are more sensitive to loud and high-tone noise [Wever EG, 1963] and their cochlear anatomy is easy to access. Unlike the situation in humans, the guinea pigs' cochleae are not hidden within the dense otic capsule bone but protrude into the middle ear space. They are located just beneath the middle ear ossicles and easily to reach by a posterior approach. In fact, various types of animals such as cats [Hinojosa R, 1966], chicken [Sliwinska-Kowalska M, 2000; Xiang ML,

2008], rabbits [Mom T, 1999], rats [McLaren GM, 1993; Fraenkel R, 2001; Fujioka M, 2006], gerbils [Boettcher FA, 1996], and chinchillas [Attanasio G, 2001] have been used in a variety of hearing research studies, but guinea pigs are used more commonly in studies of cochlear microcirculation and stimulation of hearing loss [Thorne PR, 1987; LaRouere MJ, 1989; Calisle L, 1990; Brown JN, 1994; Lamm K, 1998; Lamm K, 2000; Itou M, 2001; Cassandro E, 2003; Chen Z, 2004; Duan M, 2008].

To realize this model, we followed most steps of cochlear preparation described by Nuttall in 1987 [Nuttall AL, 1987]. We experienced that using this procedure, it required less than 30 min to access the stria vascularis microcirculation. However, it was impossible to preserve the ossicular chain during the access to the lateral portion of the cochlea by a posterior approach. The tympanic membrane, posterior annulus, and tensor tympani tendon needed to be removed as well. The techniques to observe and measure the cochlear microcirculatory parameters by using IVM are well established [Nuttall AL, 1987]. Elevation of a thin bony fragment which covered the membranous cochlea was a critical step that had to be performed with great caution. Automated bone-drilling instruments should not be used because it might directly affect the strial blood flow. According to the author's experience, the main difficulty might arise during the observation due to obstructed vision by minor bleedings. Therefore, it is advisable to keep a mastoid cavity and the cochlea window clean from any active bleeding as much as possible. Particularly when ossicular chains were removed, great care had to be taken to avoid injury to the stapedial artery.

Although a sacrifice of surrounding structures and moderate skills are necessary for the surgical procedure, IVM is an excellent method to evaluate cochlear microcirculation in animals. It had been demonstrated that microdissection techniques do not alter the cochlear blood flow [LaRouere MJ, 1989]. The advantages of IVM comparing with other methods such as laser Doppler flowmetry, reactive or labeled microsphere injection techniques, oxygen microelectrodes, and magnetic resonance imaging are the abilities to: (1) measure the velocity of red blood cells, (2) study the dynamics of microcirculatory changes, (3) define the exact region of interest, and (4) investigate morphological and physiological changes within single visualized vessels. Moreover, the flow rates provided by IVM are more precise and rather sensitive to cochler blood flow changes than the values provided by laser Doppler flowmetry [LaRouere MJ, 1989; Fuzisaki Y, 1993].

Concerning its ability to visualize specific regions directly through a microscope, single vessels could be selected and followed throughout the whole observation period. In our study, we measured diameters and velocities of at least 6 vessels per ROI, and determined the changes of microcirculatory parameters in the same respective vessels. However, it is not possible to measure the hearing threshold and microcirculatory changes in the same ear. Once the cochlea bone is opened, some unavoidable leak of perilymph might as well cause hearing threshold increase and confound the hearing results. For this reason, we decided to measure hearing thresholds on the contralateral ear but at the same time as microcirculatory measurements.

To examine acute effects of loud noise on the changes of cochlear microcirculation and hearing function, we chose the intensity of 106 dB SPL noise for 30-min duration as an exposure stimulus since there was evidence showing that cochlear blood flow can decrease by high intensity sound (~105 dB) [Scheibe F, 1993]. As described by Thorne and Nuttall [Thorne PR, 1987], a slight decrease in blood flow after the onset of 103 and 110 dB SPL noise exposure is found within 20 min, but not after application of 90 dB SPL. Scheibe and colleagues [Scheibe F, 1993] found no change of cochlear blood flow after exposure to lower intensities of 85 to 105 dB SPL. It is well known that there is a correlation of certain areas of perfusion with specific frequencies of hearing in the cochlea. For example high frequent noise over 10 kHz given with high intensity corresponds to blood flow decrease only in basal turns of the cochlea [Thorne PR, 1987; Okamoto A, 1992]. Hence in our study, we identified an alteration of segmental blood flow in strial capillaries located at the second turn of cochlea after exposure to 106 dB SPL centered at 4 kHz noise.

According to Lamm and Arnold [Lamm K, 1996] who also used 106 dB noise as an exposure stimulus but measured the cochlear blood flow with the laser Doppler method, a significant reduction of cochlear blood flow is not observed before *90 min* after exposure. Hence, even if we managed to record the first IVM picture of cochlear capillaries approximately 60 min after noise exposure, we did not have to expect to miss any important changes. It is in line with these considerations that initial segmental blood flow recorded at *60 min* did not differ significantly between the two groups.

Despite knowing that the greatest threshold shift usually happens at the frequency 4 kHz in human patients with NIHL, we nevertheless used the stimulus to measure response threshold at 8 kHz in this study. The reasons were the susceptibility of guinea pig ears to sound at high frequencies and the limitations of the ABR device. A dip or notch at 4 kHz, or at 6 kHz, is a

typical feature of NIHL in humans. However, this is different in guinea pigs. According to evidence from Thorne and Nuttall (their Figure 4A) [Thorne PR, 1987], the maximum threshold shift in guinea pigs following the 4 kHz-noise exposures occurs at 4, 6 and 8 kHz using ABR testing. In our preliminary trial (data not shown), we tried to measure the guinea pigs' hearing threshold at different frequencies, both before and after noise exposure. We discovered that measuring tone bursts-ABR at 8 kHz gave the best waveform responses. Measuring before noise exposure, the quality and amplitude of the ABR wave III was easily identified with the 8 kHz stimulus. ABR wave III is the most reliable waveform in guinea pig ABR, distinctly from the ABR wave V in humans, In addition, the greatest loss of ABR threshold after loud noise was observed at 8 and 6 kHz respectively.

By using IVM and ABR for *in vivo* analysis of cochlear microcirculation and hearing function after loud noise, we could analyze in detail effects and kinetics of rheological treatments targeted at inner ear microcirculation with regard to the hearing function. The next step was to apply this model to investigate the effects of blood flow restoring agents for an improvement of hearing function in the treatment of acute NIHL.

1.2 Cochlear microcirculation and hearing function after exposure to loud noise

We showed that hearing threshold shifted significantly since *60 min* after loud noise exposure and tended not to decrease further beyond timepoint *150 min*. In contrast, segmental blood flow in cochlear capillaries decreased further until the end of our observation period (*210 min*). These measurements confirm that exposure to high-intensity noise can induce a decrease in cochlear microcirculation and is accompanied by an impairment of hearing function.

Using IVM, we were able to visualize capillary networks in the cochlear lateral wall directly. We measured diameters and velocities of a single vessel in at least 6 vessels per ROI. All changes in microcirculatory parameter were specifically measured repetitively in the same respective vessels during the observation period. The average diameter of all observed vessels in our study was 9.1 ± 0.8 µm, corresponding to the anatomical size of capillaries. In contrast, diameter of the SMA is about 61 ± 2 µm [Gruber DD, 1998]. The size measured in this model is relatively close to 9.3 µm or 12.2 µm diameters reported by Nuttall [Nuttall AL, 1987]. Even though Nuttal et al. categorized these visible vessels into spiral ligament capillaries and strial capillaries and concluded that the strial capillaries are larger in

size and allowing lower velocites than the spiral ligament capillaries, there was no clear cut-off between them in our observations. In our model, it was not possible to distinguish between these two kinds of vessels. Comparably, average V_{RBC} observed in our control animals appeared to be in accordance with mean strial vessel velocity of 112 μm/s measured by Pearlman and Kimura [Perlman HB, 1962], and within the range of mean red cell velocity in the spiral ligament vessels (120 ± 60 μm/s) measured by Nuttall et al.

For hearing threshold, it is difficult to compare the mean values with other studies, because the exposure stimulus and animal subjects differ in each study. For example, it is reported that rats and mice are more sensitive to impulse noise than guinea pigs [Duan M, 2008]. Furthermore, the irregular features of baseline hearing threshold in each animal might represent individual variations of hearing perception. In our study, the mean ± S.D. of maximum hearing threshold following the 4 kHz-centered noise was 45 ± 4.5 dB SPL with a maximum threshold shift of +25 dB SPL. This value is nearly similar to the mean ± S.D. of 48.9 ± 4.12 dB, which was measured by Thorne and Nuttall [Thorne PR, 1987] after 1 hour exposure to 103 dB SPL noise. Our measurements were in accordance with Attanasio et al. [Attanasio G, 2001], although intensity and duration of exposure were different. Our findings were also comparable to the results of Slinwinska-Kowalska et al. (1992) and Yamasoba et al. (2005), where loud noise causes hearing threshold shifts varying from 25 to 65 dB [Sliwinska-Kowalska M, 1992] and 25 to 45 dB [Yamasoba T, 2005]. Taken together, loud noise can shift hearing levels in our experimental animals from normal to moderate degree of hearing losses within 3 hours after loud noise exposure.

Our findings confirm the hypothesis that exposure to high intensity noise can reduce blood flow in the inner ear, combined with a decrease of hearing function. Many authors believe that NIHL might be caused by mechanical destruction of hair cells and supporting structures of the organ of Corti. Recently, growing evidence shows that an increase of free radical formation in mitochondria also plays an important role in NIHL. It is believed that high-intensity noise can inhibit ATPase activities and increases oxidative stress, particularly in the cochlear lateral wall. The intense metabolic activity subsequently increases mitochondrial free radical formation in the inner ear [Lim DJ, 1971; Yamane H, 1995; Ohlemiller KK, 1999]. Noise-induced free radical formation is thought to be a significant factor in cochlear blood flow reduction [Nuttall AL, 1999; Quirk WS, 1995]. As stated in most recent reviews, there is a causative relationship between the elevated ROS production and a reduced cochlear blood flow, particularly at the tissues of cochlear lateral wall where the cochlear vasculature is located

[Le Prell CG, 2007]. ROS peroxidises lipids that exert formation of potent vasoconstrictors, for example, 8-isoprostane-F2α (8-iso-PGF2α) [Ohinata Y, 2000], as well as reduces natural bioavailability of NO [Wolin MS, 1996]. In addition to vasoconstriction, sludging of blood cells in the cochlear lateral wall and spiral lamina vessels is observed in noise-damaged cochleae, as well [Nakai Y, 1988]. Since there was no change in diameter of strial capillaries after loud noise in our study, we assumed that vasoconstriction exists elsewhere in the feeder vessels such as the branching arterioles from SMA.

Under physiological condition, the appropriate blood flow is maintained by strictly balanced autoregulation. But in NIHL, this balance is altered and sufficient to interrupt adequate supplies of O_2 and nutrients and/or elimination of waste products from cochlear cells [Quirk WS, 1995; Seidman MD, 1999; Hawkins JE, 1971]. This results in ischemia of cells within the stria vascularis and eventually inability to maintain the endocochlear potential which is necessary for hair cells transduction. Since labyrinthine function is closely related to proper homeostasis, impairment of cochlear microcirculation can thus lead to significant hearing dysfunction [Seidman MD, 1999]. In our study, acute impairment of hearing threshold was detected within 1 hour after loud noise and slowly increased up to about 45 dB SPL over a 3 h period accompanied by a rapid and continuous decrease in blood flow in strial capillaries. When segmental blood flow was reduced by -44%, hearing threshold increased up to +23 dB.

2. Effects of TNF-α inhibition on cochlear microcirculation and hearing function in acute NIHL

Although NIHL is often reversible, the possibility of permanent hearing loss is unpredictable and there is no definite treatment for NIHL thereafter. However, better understanding of these pathways has driven the otologists to multiple concepts of therapeutic interventions. Several agents have been proposed in order to restore or prevent hearing dysfunction from noise-challenge. Among them, antioxidants or scavengers of free oxygen radicals and vasoactive drugs have been studied most frequently focusing on otoprotective outcomes. Major drawback of many experimental studies is therapy begins right before noise exposure. As shown in previous experiments, excessive noise can acutely damage the inner ear and weaken hearing ability by reducing cochlear blood flow. In this section, we investigated the effects of TNF-α inhibition in acute NIHL and found out that etanercept can restore hearing function by maintaining cochlear blood flow.

2.1 Anti-TNF-α therapy for acute NIHL

Etanercept is a TNF-α inhibitor which acts like TNF-receptor analog. Compared with other TNF antagonists such as adalimumab and infliximab, etanercept has the best characterized pharmacokinetic properties [Nestorov I, 2004]. Etanercept is approved as an alternative therapy for patients with autoimmune diseases, such as rheumatoid arthritis, juvenile rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis [Mease P, 2001], because it was shown to be more effective and better tolerated than common methotrexate treatment [Chen YF, 2006]. In otolaryngology, there have been reports of using TNF-α inhibitors in autoimmune or inflammatory-related diseases, for example, immune-mediated inner ear disease [Wang X, 2003; Staecker H, 2002; Cohen S, 2005; Matteson EL, 2005; Lobo D, 2006; Van Wijk F, 2006], sudden sensorineural hearing loss [Street I, 2006], otitis media with effusion [Kim DH, 2006], and Wegener's granulomatosis with head and neck manifestations [Erickson VR, 2007]. So far, most of successful treatments have been reported from experiments in animals, and currently, there is no etanercept treatment used in clinical routine.

Although suppression of immune responses by inhibiting TNF-α signal has been demonstrated to reduce hearing loss in inner ear studies [Satoh H, 2002; Staecker H, 2002; Wang X, 2003; Matteson EL, 2005; Street I, 2006], the exact mechanism and function of TNF-α in NIHL is poorly understood. Apart from ROS and isoprostane formation following loud noise, it is possible that there are other potent vasoconstrictors associated in cochlear blood flow reduction. TNF-α is one of the most important proinflammatory cytokines mediating inflammation particularly in early phases. As demonstrated by Fujioka [Fujioka M, 2006], TNF-α is the earliest cytokine increased within few hours after the onset of noise exposure. Based on recent findings, production of ROS can stimulate the cytokine cascade through NF-χB-induced transcriptional events, which can induce the expression of TNF-α [De Martin R, 2000; Ye J, 2003]. In contrast, TNF-α can stimulate further ROS production, such as peroxynitrite and superoxide radicals (O_2^-), by upregulation of NAD(P)H oxidase [Sorescu D, 2002] and iNOS [Pritchard Jr K A, 1995; Cai H, 2000]. Moreover, TNF-α is also a potent activator of the enzyme Sk1, which produces S1P, another significant vasoconstrictor for SMA [Scherer EQ, 2006].

After reviewing data from the literatures, there are two main concepts that support the important role of TNF-α on NIHL.

The first rationale is based on the concept of cochlear ischemia and impairment of cochlear blood flow after loud noise, as well as the fact that TNF-α evokes vasoconstriction of SMA:

(1) TNF-α increases calcium sensitization of the contractile apparatus by activation of Sk1 with impact on the S1P/$S1P_2$ and on the Rho/Rho kinase pathways.

As shown by Jayaraman et al. [Jayaraman T, 2008], highly expression of TNF-α correlates with increased activity of calcium channels that regulate intracellular calcium of vascular smooth muscle cells. TNF-α activates the enzyme Sk1 to produce S1P from sphingosine. On the cell surface, S1P forms ligand bindings to specific S1P receptors and subsequently induces vascular smooth muscle contraction by increasing intracellular calcium sensitivity by the Rho/Rho kinase pathway [Bolz SS, 2003]. Latest evidence also supports the essential role of the S1P/$S1P_2$ receptor system on hearing function [Kono M, 2007].

2) TNF-α may reduce NO-mediated vasodilation by diminishing the production of NO and enhancing the removal of NO [Zhang H, 2009].

By reduced production of NO, TNF-α is shown to suppress not only eNOS expression but also the availability of arginine (a precursor for NO) in endothelial cells [Picchi A, 2006; Goodwin BL, 2007]. During ischemia-reperfusion, TNF-α increases expression of arginase, an enzyme that serves as an endogenous eNOS competitor for arginine [Gao X, 2007]. *In vitro* TNF-α can increase ROS production by upregulating NAD(P)H oxidase, resulting in increased removal of NO from vascular smooth muscle cells [Gao X, 2007]. According to Ungvari et al. [Ungvari Z, 2003] and Keller et al. [Keller M, 2006], TNF-α as well as S1P can induce ROS generation, upregulate NAD(P)H oxidase and iNOS, but reduce bioavailability of NO. When blood flow to stria vascularis is reduced, the strial cells cannot function properly, and therefore fail to maintain K^+ level in the endolymph. Finally, the loss of the endocochlear potential leads to poor hair cells transduction and subsequent hearing loss.

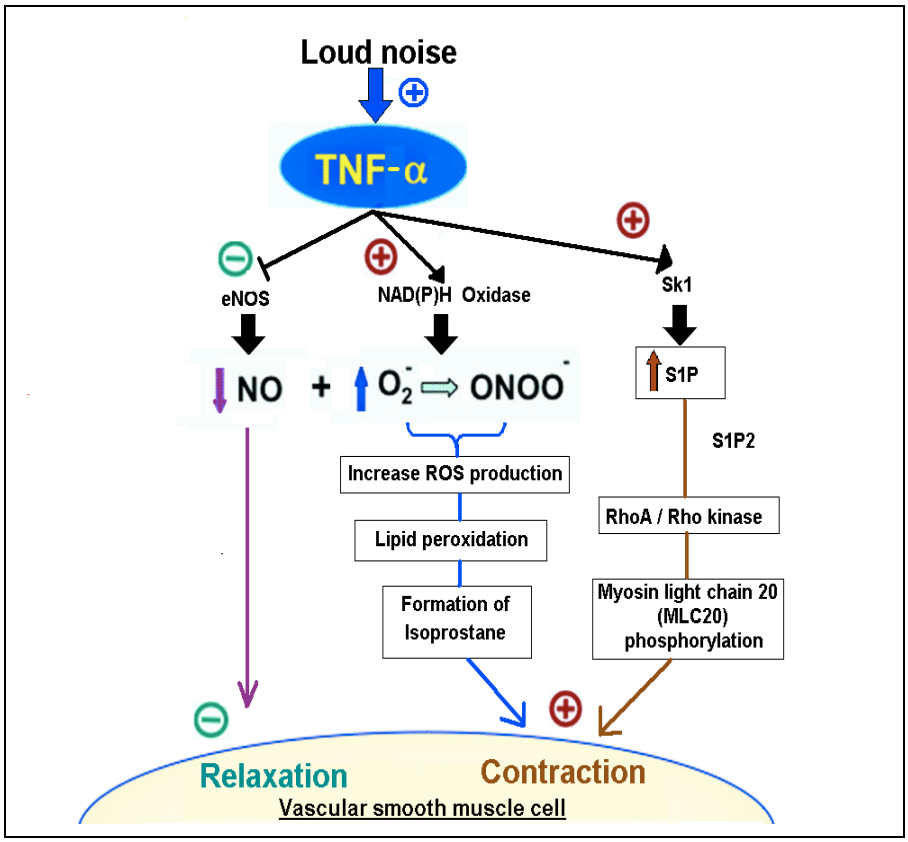

Figure 31 *In NIHL, TNF-α possibly reduces cochlear blood flow by; (1) reducing the bioavailability of NO to induce SMA smooth muscle relaxation, and (2) increasing the formation of vasoconstrictors such as 8-isoprostane-F2α and S1P. TNF-α reduces NO production via down regulation of eNOS activity. Furthermore, TNF-α upregualtes the NAD(P)H oxidase which turns NO into peroxynitrite ($ONOO^-$). As a consequence, levels of ROS increase. Peroxynitrite is a potent oxidant that can oxidize phospholipids and forms 8-isoprostane, which possesses potent smooth muscle contractive activity during noise-overstimulation [Praticò D, 2001; Ohinata Y, 2003; Miller JM, 2003]. Moreover, TNF-α might also participate in the Sk1/S1P controlled SMA vasoconstriction signaling pathway because it is a potent activator of the enzyme Sk1. After sphingosine is phosphorylated to S1P by Sk1, it forms ligand bindings with $S1P_2$ receptors and stimulates calcium sensitivity of the contractile apparatus of vascular smooth muscle cells via Rho / Rho kinase and MLC phosphorylation [Büssemaker E, 2007].*

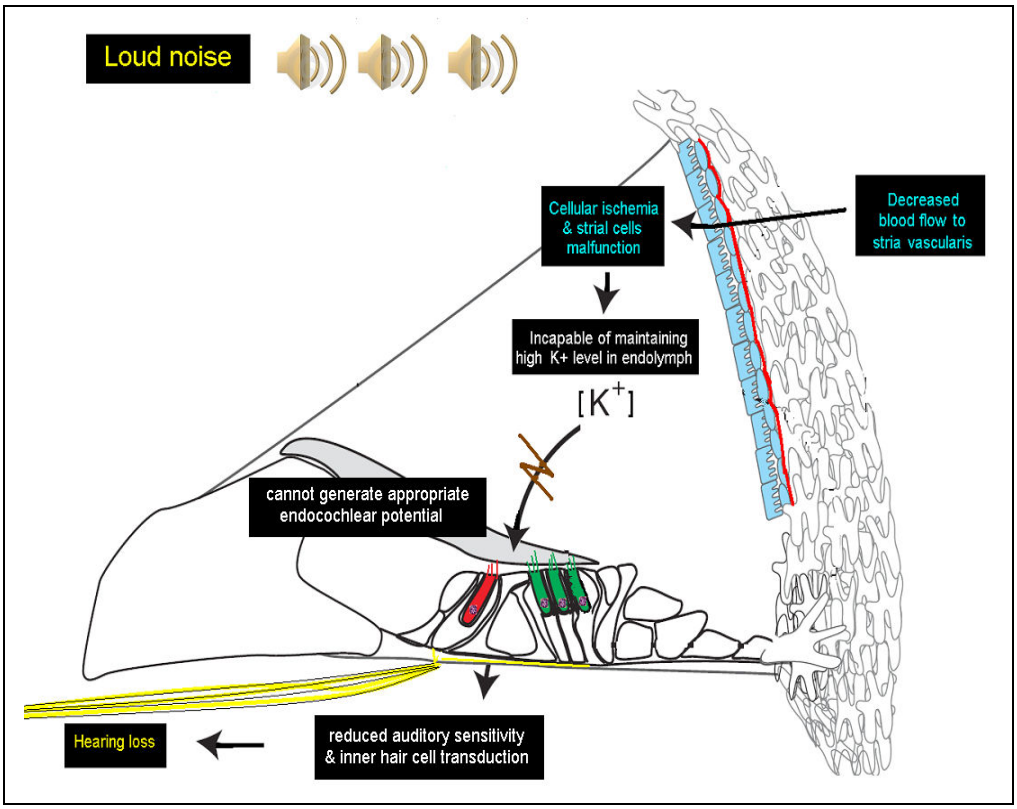

Figure 32 *Schematic synopsis of the linkage between inner ear microcirculation and hearing function. Following the effect of loud noise on SMA vasoconstriction, the reduced blood flow to cochlea (particularly in the area of stria vascularis) results in strial cells ischemia. Strial cells cannot function properly, and fail to maintain K^+ levels in the endolymph. Finally, the loss of endocochlear potential leads to poor hair cell transduction and subsequent hearing loss.*

The second rationale is not related directly to cochlear blood flow but rather the action of TNF-α in inflammation. TNF-α mediates initial inflammatory responses particularly mediated by spiral ligament fibrocytes which interconnect the basal cells of stria vascularis with gap junctions. When the cultured fibrocytes were stimulated by TNF-α, they secrete chemokines and other mediators that induce recruitment of inflammatory cells [Ichimiya I, 2000; Tornabene SV, 2006; Keithley EM, 2008] as well as the production of many toxic substances including ROS. The cochlea, an organ with limited regeneration capacity, can be significantly degenerated by inflammatory cells leading to permanent destruction and hearing loss [Ma

C, 2000; Satoh H, 2002]. According to this, TNF-α might also cause NIHL due to:

(1) Direct disruption of the spiral ligament fibrocytes and edema of stria vascularis [Ichimiya I, 2000; Hirose K, 2003], which therefore directly affect the capability to maintain K+ concentration and the endocochlear potential.
(2) Further accumulation of ROS in outer hair cells. When outer hair cells are damaged or lost, the threshold sensitivity of the inner hair cells increases (loss of active cochlear amplification) [Lynch ED, 2005].
(3) Induction of hair cell apoptosis by activation of kinases of the stress-activated protein kinase/JNK pathway [Shaulian E, 2002; Wajant H, 2003]. Positive staining of phosphorylated JNK was found in the organ of Corti within 3 hours after impulse noise and before presence of cells with fragmented DNA [Murai N, 2008].
(4) Induction of vascular endothelial cell expression of soluble intercellular adhesion molecule 1 (ICAM-1) [Stannard AK, 2007], which allows more extravasation of circulating monocytes and lymphocytes to inflammed site [Springer TA, 1987; Tornabene SV, 2006], thereby leading to endothelial wall dysfunction [Ross R, 1999]. This mechanism may be similar to what happens in sudden sensorineural hearing loss due to autoimmune disorders [Quaranta N, 2008].

The question was raised whether anti-TNF-α therapy not only enhances cochlear microcirculation during noise-overstimulation, but also prevents permanent cochlear damage by blocking signal transduction inflammatory pathways. With regard to the data derived from the literature, it is reasonable to consider a TNF-α inhibitor - such as etanercept - in order to restore cochlear function and prevent permanent threshold shift.

2.2 Changes of cochlear microcirculation and hearing function after etanercept administration in acute NIHL

The establishment of an experimental model of acute NIHL using IVM and ABR enables precise, real-time dynamic and quantitative measurements of changes of cochlear microcirculation and hearing function. Exposure to loud noise led to remarkable cochlear blood flow reduction and ABR threshold shift, within 90 min after exposure. Furthermore, improvement of cochlear blood flow and hearing function were observed after administration of etanercept. The findings differed significantly from control (NSS-injected) animals where segmental blood flow was impaired and hearing thresholds were raised. Considering therapeutic windows, observations in this study favour satisfactory hearing restoration when animals were treated early. Since the elevated levels of TNF-α remain in the cochlea up to 6 h after noise exposure [Fujioka M, 2006], we suggest that treatment with a TNF-α inhibitor should be delivered within this interval. In our study, we injected etanercept within 90 min after animals were exposed to noise and found that etanercept action still improved cochlear blood flow immediately after the onset of administration. Systemic therapy with etanercept elevated cochlear blood flow significantly within 15 min. When segmental blood flow was reduced by -36.0% in control animals, almost no reduction (-2.2%) was observed in the etanercept-therapy group. Similarly to hearing thresholds, when total threshold shift was as much as +20 dB SPL in control animals, there was also no threshold shift subsequent to etanercept administration (-0.8 dB SPL).

It is possible that the effectiveness of the vasoactive therapy in NIHL relies exclusively on the early onset of treatment. To protect auditory function, restoration of impaired microcirculation must be provided before ischemia of cochlear tissue exerts irreversible consequences. According to Pearlman et al. [Perlman HB, 1959], Thalmann et al. [Thalmann R, 1972], and Tabuchi et al., acute ischemia can cause tissue anoxia and deteriorate cochlear action potentials already within 1 h. It has also been reported that hearing improvement of patients with acute noise injury was significantly better when patients were treated by prednisolone and piracetam within the first hour after loud noise but not later [Psillas G, 2008]. Meanwhile, post-noise treatment with methylprednisolone, the agent that also possesses vasoactive property, after 3 h tends not to exhibit any favorable hearing outcome as seen in immediate post-noise exposure treatment [Tabuchi K, 2006]. Furthermore, single or acute administration of vasoactive drugs after exposure to loud noise may be adequate because there was no further

progression (worsening) of hearing threshold shift on the second day following noise overstimulation and treatment [Psillas G, 2008].

This is the first study that clearly shows TNF-α inhibitors as blood flow restoring substance in acute NIHL. Most of the recent studies suggest that anti-TNF-α therapy provides improvement of inflammatory-related endothelial dysfunction under other conditions. For example, in inflammed middle ear mucosa, antagonizing TNF-α activity by TNF soluble receptor type I (sTNFRI) can reduce capillary permeability, subepithelial edema, and inflammatory cell infiltration [Kim DH, 2006]. In the cochlear lateral wall, infiltrating inflammatory cells are significantly reduced after the animals have been systemically treated with etanercept [Satoh H, 2002]. In addition, TNF-α inhibitors significantly decreased free radicals production in coronary artery smooth muscles [Ungvari Z, 2003]. However, regarding autoimmune hearing loss, the successful treatment of TNF-α inhibitors on cochlear function is still controversial. It seems that etanercept is as effective as glucocorticoids [Lobo D, 2006] in hearing improvement but patients not always recovered from hearing loss [Matteson EL, 2005]. Patients with autoimmune inner ear disease did not gain hearing levels after treatment with etanercept. The only advantage of etanercept treatment in these patients appeared to be stabilization of hearing and thus no further progressive hair cell loss [Matteson EL, 2005].

Despite promising outcomes, the reports of favourable hearing restoration following etanercept administration have been accomplished only in animals and are currently still in the initial phase of clinical trials. Further studies regarding the exact type of TNF-α inhibitors, dosage, as well as route of administration must be designed thoroughly before considering this therapy for patients with acute NIHL. Even though etanercept is reported to be well tolerated by most patients [Matteson EL, 2005], serious adverse effects such as increased risk of infections (e.g. pneumonia, tuberculosis, cellulitis, bacterial sepsis), malignancies (e.g. lymphoma, basal cell carcinoma), pancytopenia, myocardial infarction, multiple sclerosis and depression [Tyring S, 2007] must be taken into consideration. Fortunately, these serious toxicities are rare and the important risks usually occur upon continuous use [Saad AA, 2008] rather than after single dosage.

V SUMMARY

Acute noise-induced inner ear hearing loss is characterized by microcirculatory disturbance in the *stria vascularis*. In addition to the immunomodulatory effect, inhibition of TNF-α activity might prevent vasoconstriction of the spiral modiolar artery by inactivation of Sphingokinase-1 in the S1P/$S1P_2$ signaling system in vascular smooth muscle cells as well as reduce downregulation of NO-mediated vasodilation. Therefore, early treatment with TNF-α-inhibitors might prevent hearing impairment by restoring cochlear blood flow.

In order to investigate acute effects of loud noise exposure on cochlear microcirculation and hearing function, we have established a new standardized animal model by using *in vivo*-fluorescence microscopy and auditory brainstem response. Fluorescent dextran as a blood plasma marker was given intravenously in guinea pigs under narcosis. On one ear, cochlea and *stria vascularis* were surgically exposed for microscopic analysis. On the contralateral ear, hearing threshold was measured by auditory brainstem response after exposure of both ears to loud noise (106 dB SPL, 30 min). Control animals were not exposed to noise. In contrast to control animals, cochlear blood flow was reduced by 44 % while the hearing threshold increased by 23 dB SPL at the end of the observation period (210 min) after loud noise exposure.

After using this model for therapeutic evaluation, early treatment with a single dose of TNF-α-inhibitor – *etanercept* - was shown to restore cochlear blood flow and maintain hearing threshold. When cochlear blood flow was reduced by 36.0 % in saline-treated control animals, only 2.2 % reduction was observed under TNF-α-inhibition at the end of the observation. Similarly, when the total hearing threshold shift reached + 20 dB SPL in control animals, there was almost no threshold shift subsequent to TNF-α-inhibition therapy.

In conclusion, these data clearly show that TNF-α-inhibition is a promising treatment strategy in acute noise-induced hearing loss.

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VII APPENDIX

1. Publication of the study

Original papers

Canis M*, **Arpornchayanon W***, Messmer C, Suckfuell M, Olzowy B, Strieth S (2010) An animal model for the analysis of cochlear blood flow [corrected] disturbance and hearing threshold in vivo. Eur Arch Otorhinolaryngol 267:197-203; * equal contributions by these two authors

Apornchayanon W*, Canis M*, Suckfuell M, Strieth S: A new animal model for in vivo analysis of cochlear microcirculation and hearing function after acute loud noise; *submitted*; * equal contributions by these two authors

Arpornchayanon W*, Canis M, Suckfuell M, Berghaus A, Strieth S: TNF-α inhibitor restores cochlear microcirculation and hearing function in acute NIHL; *manuscript in preparation*

Oral presentation

Arpornchayanon W, Canis M, Suckfuell M, Strieth S: Tiermodell zur Analyse von kochleärer Mikrozirkulation und Hörfunktion bei Lärmtraumen.
(59. Tagung der Oto-Rhino-Laryngologischen Gesellschaft zu München e. V., 6-7.12.2008, München)

Arpornchayanon W, Canis M, Suckfuell M, Strieth S: Analysis of cochlear microcirculation and hearing function *in vivo.*
(Kolloquium über Experimentelle Pathophysiologie im Institut für Chirurgische Forschung München, 08.12.08)

Strieth S, Canis M, Suckfüll M, **Arpornchayanon W**: Ein neues Tiermodell zur simultanen Analyse von cochleärer Mikrozirkulation im Bereich der Stria vascularis und der Hörfunktion nach akuter Lärmschädigung des Innenohres.

(9. Jahrestagung der Norddeutschen Gesellschaft für Otorhinolaryngologie und zervikofaciale Chirurgie/ 2. German Polish ENT Symposium, 12.06.-13.06.2009, Hannover)

Strieth S, Canis M, Suckfüll M, **Arpornchayanon W**: Ein neues Tiermodell zur Analyse von cochleärer Mikrozirkulation und der Hörfunktion nach akuter Lärmschädigung des Innenohres.
(93. Jahrestagung der Vereinigung der Südwestdeutschen Hals-Nasen-Ohrenärzte, 17.09.-19.09.2009, Neu-Ulm)

Strieth S, Canis M, Suckfuell M, **Arpornchayanon W**: Ein neues Tiermodell zur Analyse von kochleärer Mikrozirkulation und Hörfunktion nach akuter Lärmschädigung des Innenohres.
(Vortrag, Jahrestagung der Vereinigung Westdeutscher Hals-Nasen-Ohren-Ärzte von 1897 im 114. Jahre des Bestehens, 19.-20.03.2010, Bochum)

Poster

Arpornchayanon W, Canis M, Suckfuell M, Strieth S: A new animal model for in vivo analysis of cochlear microcirculation and hearing function after exposure to loud noise; 24.-27.09.2008, Aachen, Germany; *J Vasc Res* in press
(Annual Meeting of the Society for Microcirculation and Vascular Biology (GfMVB) 2008, 25-27.09.2008, Aachen)

2. Acknowledgement

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Finally, I am thankful to all members in the committee for their time and energy that they have devoted to read my work.

3. Curriculum vitae

Personal data

Name:	Warangkana Arpornchayanon
Date of birth:	April 2, 1979
Birthplace:	Chiang Mai, Thailand
Sex:	Female
Nationality:	Thai
Status:	Married

Educational background

1985 – 1990	Regina Coeli College
1991 – 1995	Chiang Mai University Demonstration School
June, 1995	Final secondary-school examination from the non-formal education of Thailand
1996 – 2002	Doctor of Medicine, Faculty of Medicine, Chiang Mai University
April, 2002	Qualification for medical practice, Thai Medical Council, Thailand
2002 – 2003	Graduate Diploma in Clinical science (Family Medicine), Faculty of Medicine, Chiang Mai University
2003 – 2004	Graduate Diploma in Clinical Science (Otolaryngology), Faculty of Medicine, Chiang Mai University
Since Oct, 2007	Work for doctoral thesis at the Department of Otorhinolaryngology, Head and Neck Surgery and the Walter-Brendel-Center of Experimental Medicine, University of Munich (LMU)

Medical training

2002 – 2003	Internship in Maharaj Nakorn Chiang Mai Hospital
2003 – 2007	Residency training in Otolaryngology, Department of Oto-rhinolaryngology, Maharaj Nakorn Chiang Mai Hospital

Since Feb, 2009 Research fellowship in Clinical pharmacology, Department of Clinical pharmacology, University of Tübingen, Germany

Licensure and Certification

April, 2002 Medical license, Thai Medical Council

June, 2007 Certifying of Proficiency in Otolaryngology, the Royal College of Otolaryngologists of Thailand and Thai Medical Council

Feb, 2009 Certificate of Investigator, Centrial, Tübingen

Professional and academic memberships

Since 2002 Member of Thai medical council

Since 2006 Member of the Pharmacological & Therapeutics Society of Thailand

Since 2007 Member of the Royal College of Otolaryngologists of Thailand

Occupational background

2002 – 2006 Hospital physician, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai University

Oct, 2008 – Jan, 2009 Scientific researcher, Department of Otorhinolaryngology, Head and Neck Surgery, University of Munich

Since March, 2006 Faculty member and instructor, Department of Pharmacology, Faculty of Medicine, Chiang Mai University

Other experience

March, 1999 Exchanged medical student at the Ryukyu University, Okinawa and Fukui Medical University, Fukui, Japan

Feb, 2001	Exchanged medical student at Nara Medical University, Nara, Japan
July, 2004	Attendant in the Otological center: Bangkok & International Center for Otologic Training Temporal Bone Surgical Dissection: Basic course
March, 2005	Attendant in Temporal bone course for Otolaryngology residents, Faculty of Medicine, Chiang Mai University
March, 2006	Attendant in Temporal bone course for Otolaryngology residents, Faculty of Medicine, Chiang Mai University
April, 2006 – June, 2007	Committee of Therapeutic drug monitoring, Clinical pharmacology unit, Department of Pharmacology, Maharaj Nakorn Chiang Mai hospital
June, 2007 – Sep, 2007	Graduate German language courses; level B1 from Goethe-Institut, Mannheim, Germany
June, 2007 – Sep, 2008	DAAD scholarship holder at the Walter-Brendel- Center for Experimental Medicine (WBex) and the Department of Otorhinolaryngology, Head and Neck Surgery, University of Munich
Feb, 2009 – May, 2009	Sub-investigator of 'First in Men' study: Safety, tolerability and pharmacokinetics of increasing single oral doses of BYK321084 in healthy male volunteers, Department of Clinical pharmacology, University of Tübingen, Germany
Since May, 2009	Investigator of 'Pharmakogenetische Faktoren bei Pharmakokinetik und Nebenwirkungen von Metoclopramid und Diphenhydramin' study, Department of Clinical pharmacology, University of Tübingen, Germany

Reserch & Written papers

Intratympanic dexamethasone for idiopathic sudden SNHL after failure of conventional therapy: a comparative study in Maharaj Nakorn Chiang Mai hospital; *Research topic during residency training*

Arpornchayanon W, Canis M, Suckfuell M, Strieth S: A new animal model for in vivo analysis of cochlear microcirculation and hearing function after exposure to loud noise; 24.-27.09.2008, Aachen, Germany; *J Vasc Res* in press)

Canis M*, Arpornchayanon W*, Messmer C, Suckfuell M, Olzowy B, Strieth S (2010) An animal model for the analysis of cochlear blood flow [corrected] disturbance and hearing threshold in vivo. Eur Arch Otorhinolaryngol 267:197-203; * equal contributions by these two authors

Schneider M, Wortmann M, Mandal PK, Arpornchayanon W, Jannasch K, Alves F, Strieth S, Conrad M, Beck H (2010) Absence of glutathione peroxidase 4 affects tumor angiogenesis through increased 12/15-lipoxygenase activity. Neoplasia 12:254-263

Olzowy B, Deppe C, Arpornchayanon W, Canis M, Strieth S, Kummer P: Quantitative estimation of minor conductive hearing loss with distortion product otoacoustic emissions in the guinea pig; *submitted*

Apornchayanon W*, Canis M*, Suckfuell M, Strieth S: A new animal model for in vivo analysis of cochlear microcirculation and hearing function after acute loud noise; *submitted*; * equal contributions by these two authors

Arpornchayanon W, Canis M, Suckfuell M, Berghaus A, Strieth S: TNF-α inhibitor restores cochlear microcirculation and hearing function in acute NIHL; *manuscript in preparation*

Private interest

Travel, Sports (swimming, cycling), Ballet, Music (Piano)