2011

Cochlear protein expression in kanamycin treated mice

Erin A. Rellinger

COCHLEAR PROTEIN EXPRESSION IN KANAMYCIN TREATED MICE

by

Erin A. Rellinger

A Capstone Project
submitted in partial fellowship of the
requirements for the degree of:

Doctor of Audiology

Washington University School of Medicine
Program in Audiology and Communication Sciences

May 18, 2012

Approved by:
Kevin K. Ohlemiller, Ph.D., Capstone Project Advisor
Keiko Hirose, M.D., Second Reader

Abstract: Experiments evaluated cochlear expression of key stress proteins in kanamycin and saline treated C57BL/6J and CBA/J mice using immunocytochemistry. A qualitative approach was used to assess immunoreactivity for HSP70, HSF-1, HO-1, and TNF-α as a function of strain and treatment.

ACKNOWLEDGEMENTS

The author wishes to express her gratitude to her mentor, Kevin Ohlemiller, Ph.D., for his guidance and mentorship through this study. The research skills that he shared were highly valued and his encouragement and teaching expertise throughout this basic science laboratory experience was very much appreciated. The amount of time he devoted to this study and training of the author over the past three years is immensely appreciated, especially given his rigorous schedule of research and academic obligations.

Patricia Gagnon, M.S. played an integral role of enabling the author to perform this study by sharing her expertise in immunocytochemistry, lab skills, animal handling and histology.

Together, Dr. Ohlemiller and Patty shared their expertise and invited me to join their laboratory which enabled me to complete this study and gain a tremendous amount of research experience over a three year time period.

The author would like to thank Pat Keller for sharing her histology knowledge and sharing skills necessary to perform this study. The time and patience that she has extended over the last couple years is greatly appreciated. Angie Schrader assisted by teaching the author the necessary imaging skills.

The author also wishes to express her gratitude to her parents, Donna and Jeff Rellinger, and sister, Alison, for their constant love and support. Thanks to Allyson Rosen, Emily Barden and Michelle Hungerford, for their friendship, companionship in the lab, and the experiences we shared; Trenton Hoffman, for the delicious meals and encouragement; and Katie Archibald and Sarah Carls for their friendship and support.

All of you enabled this capstone experience and helped it be an enjoyable one.

This Capstone Research Project was supported by:
The Department of Otolaryngology at Washington University in St. Louis School of Medicine

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABBREVIATIONS

ABR	auditory brainstem response
B6	C57BL/6J
DAPI	4’,6-diamidino-2-phenylindole
dB SPL	decibel sound pressure level
EDTA	ethylene diamine-tetra acetic acid
g	gram
HO-1	heme-oxygenase-1
HSF-1	heat shock factor-1
HSPs	heat shock proteins
i.p.	intraperitoneal
JAX	The Jackson Laboratory
kg	kilogram
KM	kanamycin
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
mg	milligram
mL	milliliter
MW	molecular weight
$NaPO_4$	sodium phosphate
PBS	phosphate buffered saline
PND	postnatal day
TNF-α	tumor necrosis factor-α
µL	microliter

INTRODUCTION

A host of studies have sought to develop pharmacotherapies against hearing loss due to noise, ototoxicity, and aging (Seidman & Vivek, 2004; Shen, Zhange, Shin, Lei, Du, Gao, Dai, Ohlemiller & Bao, 2006; Canlon, Henderson & Salvi, 2007; Le Prell, Yamashita, Minami, Yamasoba & Miller, 2007). One promising strategy seeks to elicit the protective cascades underlying preconditioning with therapeutic agents. In preconditioning, exposure to a non-damaging or minimally damaging stressor provides protection against later, potentially permanently injurious events. In preconditioning, multiple intrinsic cell defense mechanisms may be engaged to help maintain homeostasis and prevent apoptosis. Cochlear preconditioning against noise injury has been shown to be engaged by stressors including hyperthermia (Sugahara, Inouye, Izu, Katoh, Katsuki, Takemoto, Shimogori, Yamashita & Nakai, 2003; Yoshida, Kristiansen & Liberman, 1999), ischemia (Myers, Quirk, Rizk, Miller & Altschuler, 1992), hypoxia (Gagnon, Simmons, Bao, Lei, Ortmann & Ohlemiller, 2007), noise exposure (Niu, Shao & Canlon, 2003; Yoshida & Liberman, 2000), mild physical restraint (Wang & Liberman, 2002), and low-dose kanamycin exposure (Fernandez, Ohlemiller, Gagnon & Clark, 2010). Many of these studies have used mouse models which offer the advantage of reduced data variance due to genetic uniformity, as well as the possibility of genetic analysis when hearing-related phenotypes are found to differ by strain (Ohlemiller, 2006).

Recent work in CBA/J and C57BL/6J (B6) mice explored the basis of cochlear preconditioning against noise injury by low-dose, sub chronic kanamycin (Fernandez et al., 2010; Ohlemiller, Rybak Rice, Rosen, Montgomery & Gagnon, In Press). Protection was found to be robust in CBA/J, but appeared not to occur in B6. The present study addresses differences in cochlear protein expression after kanamycin (KM) treatment that might help explain these

strain differences.

Key mediators of preconditioning

A number of key mediators of preconditioning are known, and have often been found to overlap with proteins that are directly upregulated by noise and ototoxic exposure. Among these are heat shock proteins (HSPs). Named according to molecular weight (e.g., HSP70 has a MW of 70 kilodaltons), heat shock proteins encompass a family of proteins found in almost all organisms. Their dramatic upregulation is a key part of a cell's stress response. Although most dramatically upregulated by heat stress, increased HSP production can be triggered by a host of stressors, including infection, inflammation, exercise, toxin exposure and hypoxia. In the unstressed cell, heat shock proteins function in protein folding and transport, signal transduction, and cell growth and differentiation (Cunningham & Brandon, 2006; Fairfield, Kanicki, Lomax & Altschuler, 2002; Taleb, Brandon, Lee, Lomax, Dillmann & Cunningham, 2008). The induction of upregulation of HSPs can inhibit apoptotic proteins, providing overall protection for the cell (Cunningham & Brandon, 2006; Taleb et al., 2008).

Heat shock proteins are activated by the major transcription factor, heat shock factor-1 (HSF-1), which functions as a regulator of stress-induced genes for heat shock proteins and other stress-responsive proteins that aid repair and restore homeostasis. HSF-1 is expressed in many unstressed tissues, including cerebellum, heart, liver, testis and kidney tissue (Fairfield et al., 2002; Taleb et al., 2008), but becomes transcriptionally active under stressful conditions (Pirkkala, Nykanen & Sistonen, 2001). Found to be activated by ischemia and hyperthermia in rats (Higashi, Nakai, Uemura, Kikuchi & Nagata, 1995), expression of HSF-1 has been shown in inner and outer hair cells, spiral ganglion cells and the marginal and intermediate layers of the

stria vascularis in both rat and mouse cochlea (Fairfield et al., 2002). Previous studies have suggested that HSP70 and HSF-1 are both necessary for hyperthermia to cause protection against aminoglycoside-induced death (Taleb et al., 2008).

HSP70, which has been shown to be induced in the inner ear in response to cisplatin and aminoglycoside exposure, hyperthermia and cochlear ischemia, is the most widely studied heat shock protein. Cisplatin exposure has been found to increase HSP70 expression in the rat cochlea, specifically in the spiral limbus, basilar membrane, lateral wall and supporting cells (Garcia-Berrocal, Nevado, Gonzalez-Garcia, Sanchez-Rodriguez, Sanz, Trinidad, Espana, Citores & Ramirez-Camacho, 2010) and the outer hair cells and stria vascularis (Oh, Yu, Song, Lim, Koo, Chang & Kim, 2000). Increased expression of HSP70 after heat shock in the hair cells and supporting cells of mouse utricles has been shown, and heat shock inhibition of neomycin-induced hair cell death has been demonstrated (Cunningham & Brandon, 2006; Taleb et al., 2008). Cochlear ischemia has been found to induce HSP70 expression in the outer hair cells of the rat cochlea (Myers et al., 1992). HSP70 expression has also been found to protect outer hair cells in the basal and middle turns of the cochlea, along with inhibiting kanamycin induced hearing loss (Taleb, Brandon, Lee, Harris, Dillmann & Cunningham, 2009).

Heme oxygenase 1 (HO-1), also known as HSP32, is also upregulated by HSF-1 and is involved in providing protection against oxidative stress by degrading heme to produce carbon monoxide, bilirubin and iron. Expression of HO-1 has been shown in the modiolus and organ of Corti of the unstressed rat cochlea, and an increase in expression has been demonstrated in the outer hair cells and marginal and intermediate cells of the stria vascularis after heat shock (Fairfield, Kanicki, Lomax & Altschuler, 2004), which correlates with previous findings of HSP70 and HSF-1 localization. Noise exposure has also been shown to increase expression of

HO-1 in the guinea pig cochlea, where staining was seen in the outer hair cells, more specifically, Hensen's cells (Matsunobu, Saton, Ogawa & Shiotani, 2009). Another study did not show expression of HO-1 in the unstressed cochlea but expression was detected in the spleen and brain positive controls (Watanabe, Oshima, Kobayashi & Ikeda, 2003).

Along with HSPs, other intrinsic mediators of preconditioning include antioxidants and anti-apoptotics (Kawamoto, Sha, Minoda, Izumikawa, Kuriyama, Schacht & Raphael, 2003; Niu et al., 2003; Van De Water, Lallemend, Eshraghi, Ahsan, He, Guzman, Polak, Malgrange, Lefebvre, Staecker & Balkany, 2004; Darrat, Ahmad, Seidman & Seidman, 2007; Le & Keithley, 2007). The mediators of preconditioning vary by tissue (Yoshida & Liberman, 2000; Sommers-child & Kirkeboen, 2002; Das & Maulik, 2003; Dirnagl, Simon & Hallenbeck, 2003; Prass, Scharff, Ruscher, Lowl, Musselmann, Victorov, Kapinya, Dirnagl & Meisel, 2003; Eisen, Fisman, Rubenfire, Freimark, McKechnie & Tenenbaum, 2004; Pasupathy & Horner-Vanniasinkam, 2005; Ran, Xu, Lu, Bernaudin & Sharp, 2005; Gidday, 2006). Some mediators may play either harmful or beneficial roles, depending on where, and in what quantities, they are produced. Among these is tumor necrosis factor-α (TNF-α), a cytokine that functions as a regulator of inflammation which can induce both cell damage and protection against apoptosis. TNF-α expression in the cochlea has been shown in both noise and otototoxic injury (Hess, Block, Huverstuhl, Su, Stennert, Addicks & Michel, 1999; Zou, Pyykko, Sutinen & Toppila, 2005; Scherer, Yang, Canis, Reimann, Ivanov, Diehl, Backx, Wier, Strieth, Wangemann, Voigtlaender-Bolz, Lidington & Bolz, 2010). As a known mediator of inflammation, TNF-α is known to affect lipid metabolism, coagulation, insulin resistance and the function of endothelial cells lining blood vessels. TNF-α production has been shown to be significantly increased through bacterial endotoxin lipopolysaccharide (LPS) treatment, which activates the release of

pro-inflammatory cytokine TNF-α (Zhao, Brooks & Lurie, 2006). Expression of TNF-α has been shown in the lateral cochlear wall and organ of Corti (Hess et al., 1999). Vibration has been found to induce TNF-α expression in numerous areas, including the reticular lamina, outer hair cells, Dieter's cells, Hensen's cells, Claudius' cells, internal sulcus cells, spiral ligament, spiral vascular prominence, cochlear vasculature, and the spiral ganglion cells (Zou et al., 2005). Excess TNF-α has been shown to alter cochlear blood flow and cause ischemic related hearing loss through studies observing rodent and human spiral modiolar arteries and spiral ligament capillaries, which supply blood to the stria vascularis. A rapid reduction of blood was shown shortly after TNF-α was infused and a correlation between TNF-α and decreased capillary diameter was found (Scherer et al., 2010). Treatment with keyhole limpet hemocyanin (KLH) in the inner ear of mice revealed an increase in TNF-α expression and the presence of inflammatory cells in scala tympani, which suggested that TNF-α plays a role in the localized cochlear immune response (Satoh, Firestein, Billings, Harris & Keithley, 2002).

Cochlear preconditioning by kanamycin

Aminoglycosides, which include streptomycin, gentamicin, neomycin, kanamycin, tobramycin and amikacin, are routinely used for the treatment of bacterial infections. While they are cost-effective and efficient, they can be ototoxic and nephrotoxic (Rizzi and Hirose, 2007; Taleb et al., 2008; Taleb et al., 2009). Aminoglycosides have been shown to remain in the inner ear for over 30 days in a serum with a half-life of approximately 3 to 5 hours, which may increase ototoxic effects since aminoglycosides lead to the activation of apoptotic signaling cascades (Taleb et al., 2008; Tran Ba Huy, Bernard & Schacht, 1986). Most research on aminoglycosides has explored the basis of its ototoxicity and its possible remediation (Matz,

1993; Schacht, 1993; Priuska and Schacht, 1997; Schacht, 1999; Perletti, Vral, Patrosso, Marras, Ceriani, Willems, Fasano & Magri, 2008; Yu, Jiang, Zhou, Tsang, Yu, Chung, Zhang, Wang, Tang & Chan, 2011). However, recent work has shown that kanamycin applied at low doses can engage a form of preconditioning against cochlear noise injury in CBA/J mice. Fernandez et al. (2010) found that repeated subclinical doses of kanamycin have a protective effect against noise induced cochlear injury (Fig. 1). In that study, 20 day old CBA/J mice received injections of kanamycin or saline (300 mg/kg) every 12 hours for 10 consecutive days and were then exposed to 30 seconds of 110 dB SPL broadband noise on the eleventh day. Ten days later, auditory brainstem response (ABR) testing revealed normal hearing thresholds in kanamycin treated mice, compared to substantial threshold shifts in saline controls.

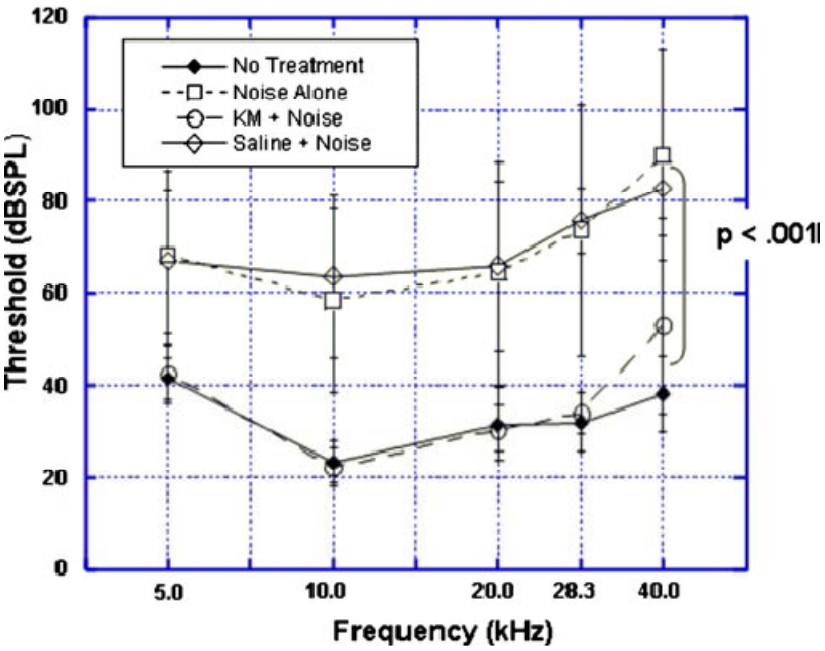

Figure 1: *Mice receiving Saline + Noise showed significantly elevated ABR thresholds versus unexposed controls. Thresholds in saline-treated mice were not significantly different from the Noise Alone group. Mice receiving KM + Noise showed ABR thresholds not significantly different from the unexposed controls.*

Reprinted with permission from Fernandez et al., 2010

Rybak Rice (2009) further investigated the shortest kanamycin dosing interval necessary to provide complete protection. Dosing regimens of once daily, once every other day, and once every third day for 10 days all provided protection. Rosen (2010) examined the effects of

genetic background on kanamycin-related protection, comparing young C57BL/6J and CBA/J mice. C57BL/6J (B6) mice were not found to exhibit the protection observed in CBA/J mice. Further studies (Ohlemiller et al., In Press), confirmed these findings, and suggested that even low-dose kanamycin is more likely to be toxic than protective in B6 (Fig. 2).

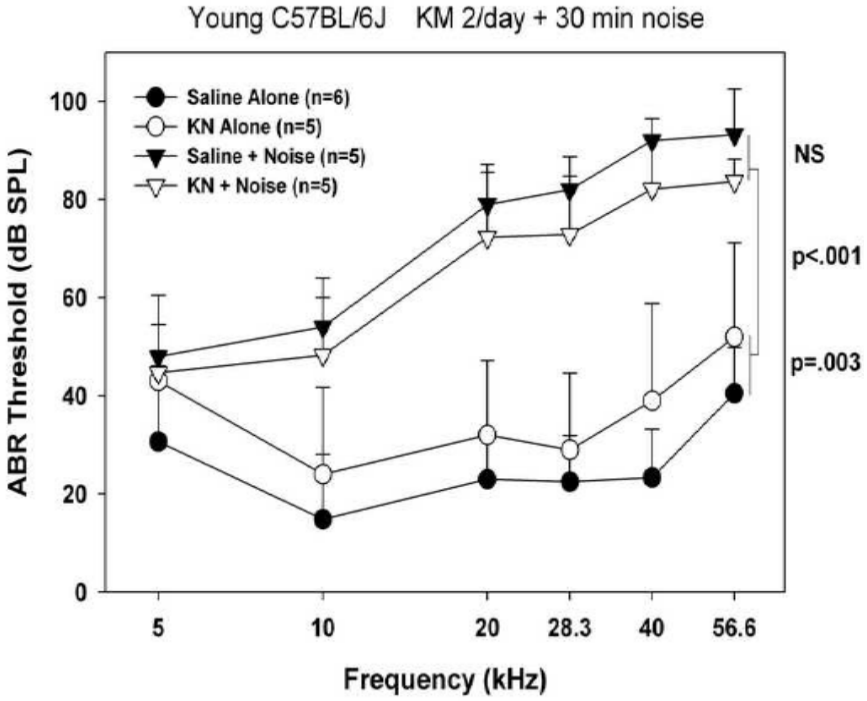

Figure 2: *ABR thresholds of KM treatment paradigm (injections twice daily) applied to young B6 mice. Unlike CBA/J, mice receiving KM alone exhibited modest (~10 dB) but statistically significant hearing loss. Also, unlike CBA/J, animals receiving KM prior to noise fared no better against noise than did saline controls. The small protective effect that might be hinted in these data may reflect protection afforded by an initial threshold shift.*

Reprinted with permission from Ohlemiller et al., In Press

To date, it has remained unclear how broadly applicable a phenomenon kanamycin preconditioning represents, since protection was not found in B6 mice. Such strain differences can be exploited, however. Phenotypic differences can be used to identify key differences in protein expression, and ultimately allelic differences in key genes that regulate protective pathways. There may be differences in metabolism, cellular uptake mechanisms, or differential expression of antioxidant enzymes between C57BL/6J and CBA/J mice that impact the ability of

kanamycin to engage preconditioning. Wu et al. (2001) have shown that genetic variability among strains plays a role in determining sensitivity to aminoglycosides. Gagnon et al. (2007) have also shown that hypoxic preconditioning against cochlear noise injury is protective in CBA/J mice, but not in B6. This raises the possibility that some common factor critical to a variety of preconditioning forms differs in form or expression between these two strains.

Purpose of the Present Study

The purpose of the present study was to investigate by immunofluorescence immunocytochemistry the role of HSP70, HSF-1, and HO-1, along with pro-inflammatory cytokine TNF-α, in kanamycin-related protection of CBA/J cochleae, as well as the possible identity of one or more of these as the basis of noted differences between CBA/J and C57BL/6J mice. Expression patterns of these prominent proteins from both the preconditioning and ototoxicity literature were examined qualitatively. Our key assumption was that any of these showing greater upregulation following kanamycin in CBA/J mice than in B6 were likely to be critical to the manifestation of preconditioning. Conversely, we posited that any of these (particularly TNF-α) showing greater upregulation in B6 mice than in CBA/J were likely to be involved in the inhibition of preconditioning.

MATERIALS AND METHODS

Animals

All CBA/J and C57BL/6J mice were derived from breeders purchased from The Jackson Laboratory (JAX) and housed in the Central Institute for the Deaf animal care facility at Washington University in St. Louis School of Medicine. The mice were housed in the

Mechanisms of Cochlear Injury laboratory in the same facility during treatment. The study used a total of 16 CBA/J mice and 17 C57BL/6J mice. All animal use and care procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

Kanamycin and saline dosing

Drug administration was randomized and each litter contained control and experimental mice of each sex. Body weight was measured twice daily. Drug treatment was tolerated, and no mice were lost. Mice received injections of kanamycin or saline vehicle every 12 hours within the same hours (±30 minutes) for a total of 10 days. Injections were administered on postnatal day (PND) 21-30. All mice were weaned on PND 25. Mice receiving kanamycin sulfate received a 300 mg/kg subcutaneous dose (63.93 mg/mL in 0.9% commercial saline solution) which was prepared every seven days. Drug dosage for each mouse was determined with the formula body weight (g) x 0.006 = injection volume (µL). Mice receiving saline received an equivalent dose dependent on their weight (300 mg/kg per dose). No noise exposure occurred; rather mice were evaluated at the time when noise would have occurred in the earlier paradigm. A total of 8 CBA/J mice received saline injections and 8 CBA/J mice received kanamycin injections. A total of 8 C57BL/6J mice received saline injections and 9 C57BL/6J mice received kanamycin injections.

Sacrifice and tissue processing for immunocytochemistry

Following kanamycin or saline injections, mice were sacrificed for immunocytochemistry on PND 31. Mice were overdosed with sodium pentobarbital (240 mg/kg, i.p.) and surgery began when there was no toe-pinch response. Transcardial perfusions were performed using a

fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer. All mice were decapitated and inspected for signs of otitis media. One animal was excluded from the study due to visible signs of middle ear pathology. Both cochleas were harvested. Cochleas were quickly removed and immersed in fixative for a total of 20 minutes and the stapes and surrounding tissue were removed while remaining in fixative. After 20 minutes, cochleas were placed in a 0.35 M Ethylene Diamine-Tetra Acetic Acid (EDTA) in 0.1 M Sodium Phosphate ($NaPO_4$) solution for 72 hours for decalcification. Then EDTA was removed and cochleas were gradually immersed in a 10% sucrose to 30% sucrose solution and remained in 30% sucrose for 24 hours at 4˚C. After 24 hours, sucrose was removed and cochleas were immersed in O.C.T. compound for 24 hours at 4˚C. Following 24 hours in O.C.T., cochleas were embedded in O.C.T. (TissueTek) in cryomolds for frozen sectioning. Right cochleas were sectioned in the mid-modiolar plane in 8µm sections utilizing a cryostat. One section from each animal was placed in random order on each slide.

Heat shock protein-70 (HSP70/HSP72)

HSP70 is not normally observed in the cochlea by immunocytochemistry (Oh et al., 2000; Cunningham & Brandon, 2006). This creates the possibility that an apparent negative result could reflect an artifactual failure to detect HSPs that are actually present. A positive control was attempted consisting of two heat shocked CBA/J mice and two CBA/J shams that were approximately 8 weeks old. The two heat-shocked mice were anesthetized with an intraperitoneal injection of a ketamine and xylazine solution (80/15 mg/kg). The mice were placed on a controlled heating pad and a rectal probe was utilized to maintain a body temperature of 41˚C for 30 minutes. After 15 minutes following the heat shock, the mice were sacrificed

utilizing the previously described protocol. Cochlear tissue and brain tissue were harvested and embedded for cryosectioning. A dilution series was performed and a dilution of 1:50 was determined to be appropriate.

Sections were soaked and rinsed in phosphate buffered saline (PBS) for 20 minutes and then incubated with a blocking solution (500 µL goat serum/50 mg Carageenan/20 µL Triton X-100/total of 10 mLs with PBS) for 120 minutes at room temperature. All incubations were performed in a humidity chamber. Sections were rinsed in PBS for 15 minutes and then incubated with the primary antibody monoclonal anti-mouse HSP70/HSP72 (Enzo Life Sciences, Lot 08021046; 1:50) for 120 minutes at room temperature. Sections were rinsed in PBS for 15 minutes and then the sections were incubated with the secondary antibody, Alexa Fluor 594 goat anti-mouse IgG (A11032, Lot 99E2-1, Molecular Probes/Invitrogen, Eugene, OR; 1:1000) for 45 minutes at room temperature. Controls were prepared with secondary antibody only; the primary antibody was replaced with PBS. Sections were then rinsed with PBS for 15 minutes, followed by a 15 minute immersion in PBS with 0.1% tween polyoxyethylenesorbitan monolaurate (Tween 20, Sigma, Lot 50K0138), and additional rinses in PBS for 10 minutes. Then sections were coverslipped with *Slow Fade*® Gold antifade reagent with DAPI (Invitrogen/Molecular Probes, Lot 734008, S36938). All sections were imaged and one section was randomly selected to display no immunoreactivity to HSP70 at a dilution of 1:50 in both C57BL/6J and CBA/J kanamycin or saline treated mice. DAPI is shown to aid in immunoreactivity localization interpretation via nuclear staining.

Heat shock factor-1 (HSF-1)

Because HSF-1 can be observed by immunocytochemistry in the normal cochlea

(Goodson and Sarge, 1995; Fairfield et al., 2002), no positive control was attempted. The previously stated immunocytochemistry protocol was utilized with a blocking solution of 500 µL goat serum/50 mg Carageenan/20 µL Triton X-100/total of 10 mLs with PBS, a primary antibody monoclonal anti-mouse HSF-1 (MAB88078, Lot LV1376830, Chemicon International, Temecula, CA; 1:100), and a secondary antibody Alexa Fluor 488 conjugated goat anti-rat IgG (A11006, Lot 52955A, Molecular Probes/Invitrogen; 1:1000). Controls were prepared with secondary antibody only; the primary antibody was replaced with PBS. All sections were imaged and one section was randomly selected to display immunoreactivity to HSF-1 at a dilution of 1:100 in both C57BL/6J and CBA/J kanamycin or saline treated mice.

Heme-oxygenase-1/Heat shock protein-32 (HO-1/HSP32)

Positive controls were provided by mid-modiolar sections of noise exposed C57BL/6J cochlear tissue and control tissue with no noise exposure and a primary antibody dilution of 1:50. The previously stated immunocytochemistry protocol was utilized in the experimental animals with a blocking solution of 500 µL goat serum/50 mg Carageenan/20 µL Triton X-100/total of 10 mLs with PBS, a primary antibody monoclonal anti-mouse HO-1 /HSP32 (OSA-1111, Lot 09010919, Assay Designs; 1:50), and a secondary antibody Alexa Fluor 594 conjugated goat anti-mouse IgG (A11032, Lot 99E2-1, Molecular Probes/Invitrogen 1:1000). Controls were prepared with secondary antibody only; the primary antibody was replaced with PBS. All sections were imaged and one section was randomly selected to display no immunoreactivity to HO-1 at a dilution of 1:50 in both C57BL/6J and CBA/J kanamycin or saline treated mice.

Tumor necrosis factor (TNF-a)

The use of a positive control was implemented using 10 mid-modiolar sections of C57BL/6J cochlear tissue treated with lipopolysaccharide (LPS). A dilution series was performed and revealed optimal staining at 1:100. The previously stated immunocytochemistry protocol was utilized with a blocking solution of 500 µL donkey serum/ 50 mg Carageenan/20 µL Triton X-100/total of 10 mLs with PBS, a primary antibody monoclonal anti-mouse TNF-α (R&D Systems, NQ13, AF-410-NA; 1:100), and a secondary antibody Alexa Fluor 594 conjugated goat anti-mouse IgG (A11032, Lot 99E2-1, Molecular Probes/Invitrogen, Eugene, OR; 1:1000). Controls were prepared with secondary antibody only; the primary antibody was replaced with PBS.

Table 1: Materials used for immunocytochemistry.

Protein	Primary Antibody	Secondary Antibody	Serum
HSP70	Hsp70/Hsp72, mAB (C92F3A-5), Lot 08021046, Enzo Life Sciences	Alexa Fluor 594 goat anti-mouse IgG, Lot 99E2-1, A11032, Molecular Probes/Invitrogen	Goat, Lot W1110, Vector Laboratories, Inc.
HSF-1	Hsf1, mAB88078, Lot LV1376830, Chemicon International	Alexa Fluor 488 goat anti-rat IgG, Lot 52955A, A11006, Molecular Probes/Invitrogen	Goat, Lot W1110, Vector Laboratories, Inc.
HO-1/HSP32	HO-1/Hsp32, mABOSA-111, Lot 09010919, Assay Designs	Alexa Fluor 594 goat anti-mouse IgG, Lot 99E2-1, A11032, Molecular Probes/Invitrogen	Goat, Lot W1110, Vector Laboratories, Inc.
TNF-α	TNFα, NQ14, AF-410-NA, R&D Systems	Cy3-conjugated AffiniPure Donkey-Goat IgG, Code 705-165-147, Lot 68618 , Jackson Immuno Research Laboratories	Donkey, Lot 68649, Jackson Immuno Research Laboratories, Inc.

Qualitative Analysis

Sections were evaluated qualitatively with a Zeiss LSM700 confocal microscope at a 20x objective in conjunction with

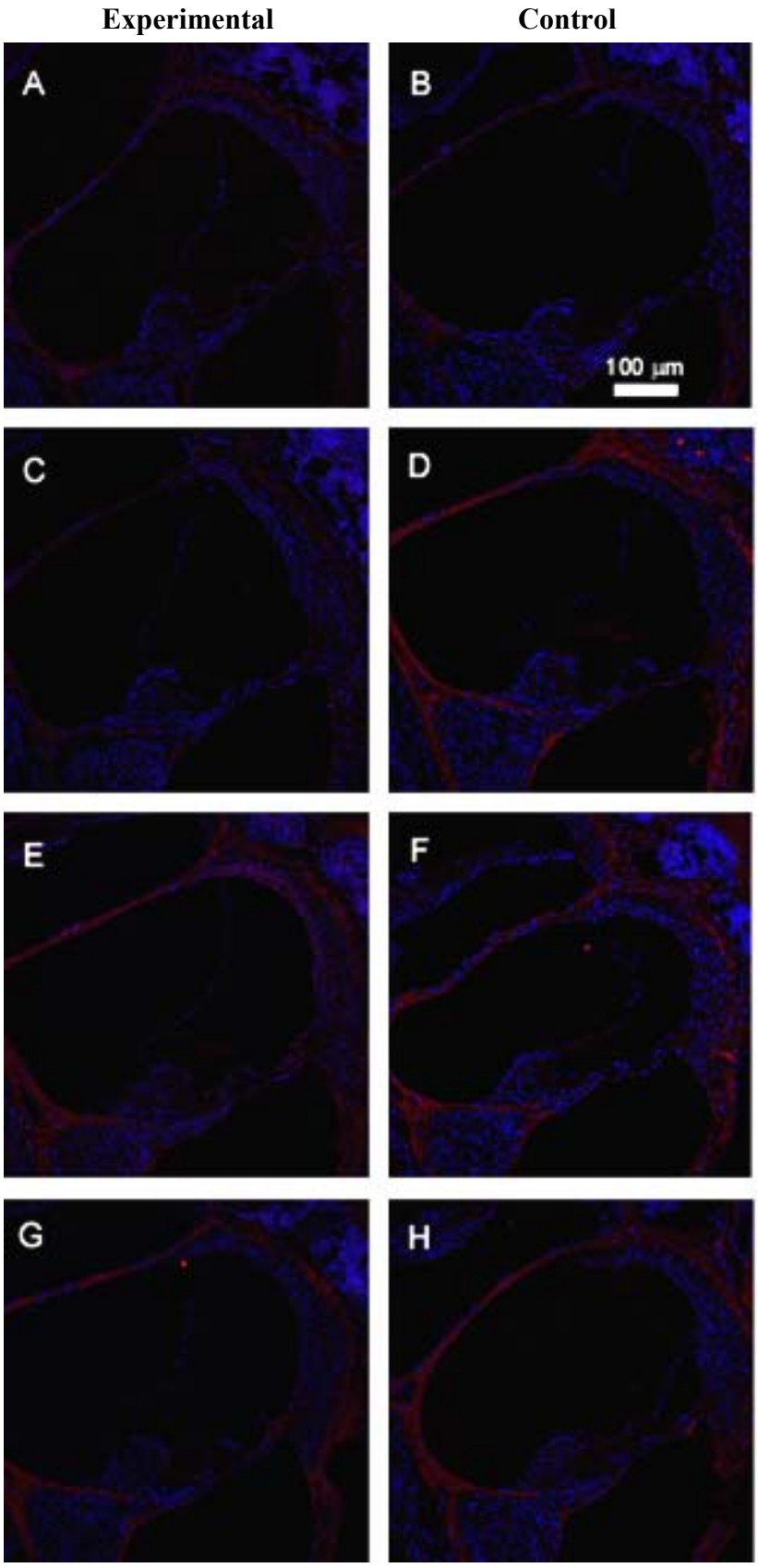

Figure 3: Cochlear upper basal turn images showing immunolocalization of HSP70. No difference in HSP70 immunoreactivity between saline and kanamycin treated B6 and CBA/J mice was observed. **(A)** Saline treated CBA/J **(B)** Saline treated CBA/J control with secondary antibody only **(C)** Kanamycin treated CBA/J **(D)** Kanamycin treated CBA/J control with secondary antibody only **(E)** Saline treated B6 **(F)** Saline treated B6 control with secondary antibody only **(G)** Kanamycin treated B6 **(H)** Kanamycin treated B6 control with secondary antibody only.

Zen softwa re. Images consist ed of the upper basal turn of the mid- modiol ar plane cochle ar section only. A determ ination of

presen
ce or
absenc
e of
each
antibo
dy was
based
on
images
digitiz
ed
using
the
same
setting
s in all
cases.

RESU
LTS

The present study revealed no expression of HSP70 in the cochlea following aminoglycoside treatment, since no difference in HSP70

immun
oreacti
vity
betwee
n
saline
and
kanam
ycin
treated
B6 and
CBA/J
mice
was
seen
(Fig.
3). It
cannot
be
confir
med
that
HSP70

was not upregu lated, but simply not detecte d, since clear immun oreacti vity was not found in brain and cochle a positiv

e
control
s.

No
expres
sion of
HO-1
as a
functio
n of
strain
(B6 or
CBA/J
) or
treatm
ent
(kana
mycin
or
saline)
was

seen (Fig. 5). Since HO-1 was detected in the stria vascularis in our samples of noise treated cochlear tissue (Fig. 6), it is likely

that we would have been able to observe significant upregulation of HO-1 by strain or condition in our kanamycin material.

Expression of HSF-1 was found in the organ of Corti, interdental cells of spiral limbus, and inner sulcus cells (Fig. 4). No difference in

HSF-1 immunoreactivity was found by strain (B6 or CBA/J) or treatment (kanamycin or saline), suggesting that similar levels of

HSF-1 were present in all conditions.

No TNF-α immunoreactivity was observed in saline or kanamycin treated CBA/J mice

or saline treated B6 mice. Greater levels of TNF-α expression were detected in B6 kanamycin treated mice compared to CBA/J kanam

ycin
treated
mice,
sugges
ting
expres
sion of
TNF-α
as a
functio
n of
genetic
strain
and
conditi
on.
The
compa
risons
betwee
n
kanam
ycin

treated B6 and CBA/J mice suppor ted that there was more TNF-α expres sed in the lateral wall (type II region and stria vascul aris) in

the B6

mice

(Fig.

10)

than

CBA/J

mice

(Fig.

8).

CBA/J
Saline

CBA/J
KM

B6

Saline

B6
KM

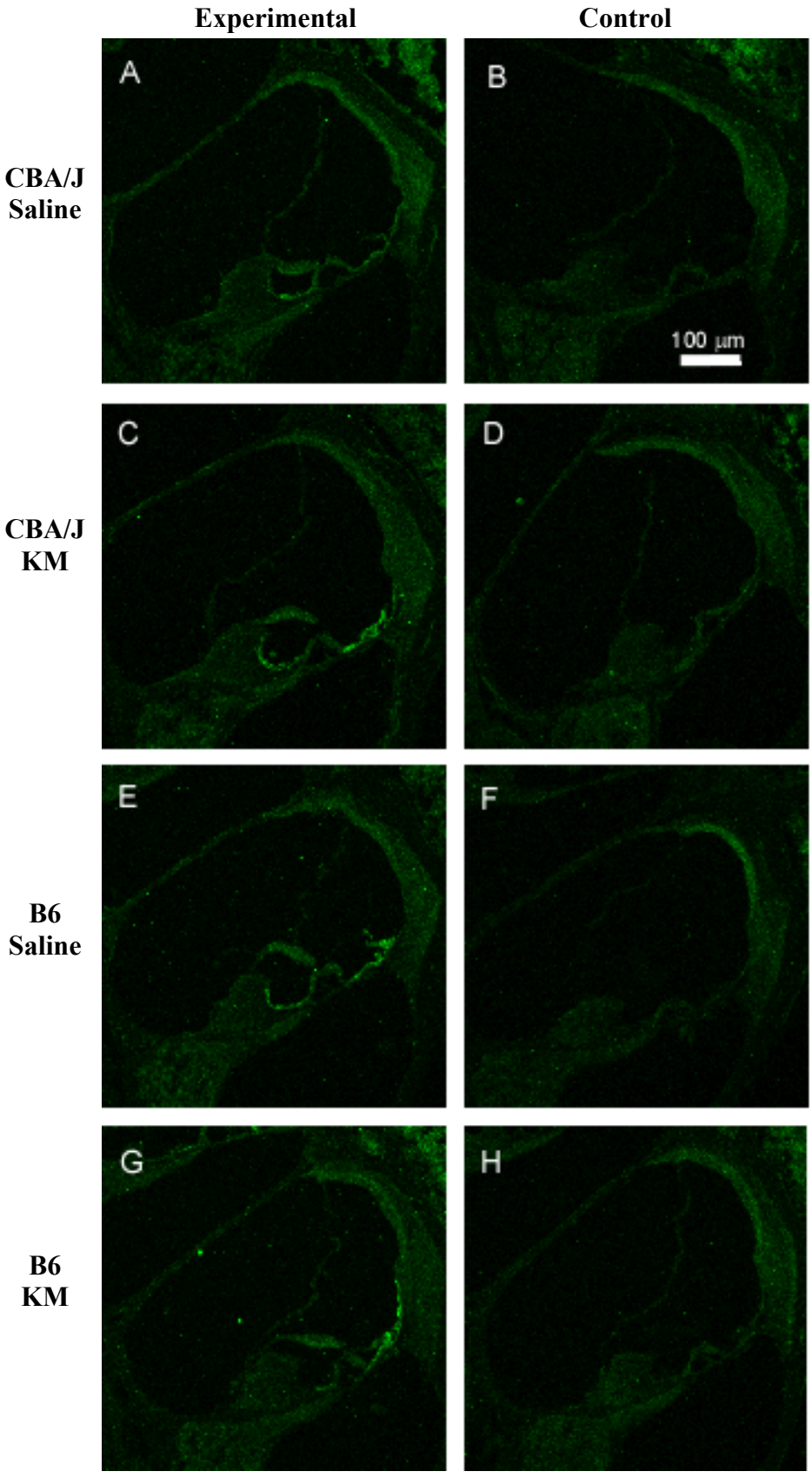

Figure 4: Cochlear upper basal turn immunolocalization of HSF-1. No difference in immunoreactivity between strain (B6 or CBA/J) or condition (kanamycin or saline) was observed. Immunoreactivity was seen in the organ of Corti, interdental cells of spiral limbus, and inner sulcus cells. **(A)** Saline treated CBA/J **(B)** Saline treated CBA/J control with secondary antibody only **(C)** Kanamycin treated CBA/J **(D)** Kanamycin treated CBA/J control with secondary antibody only **(E)** Saline treated B6 **(F)** Saline treated B6 control with secondary antibody only **(G)** Kanamycin treated B6 **(H)** Kanamycin treated B6 control with secondary antibody only.

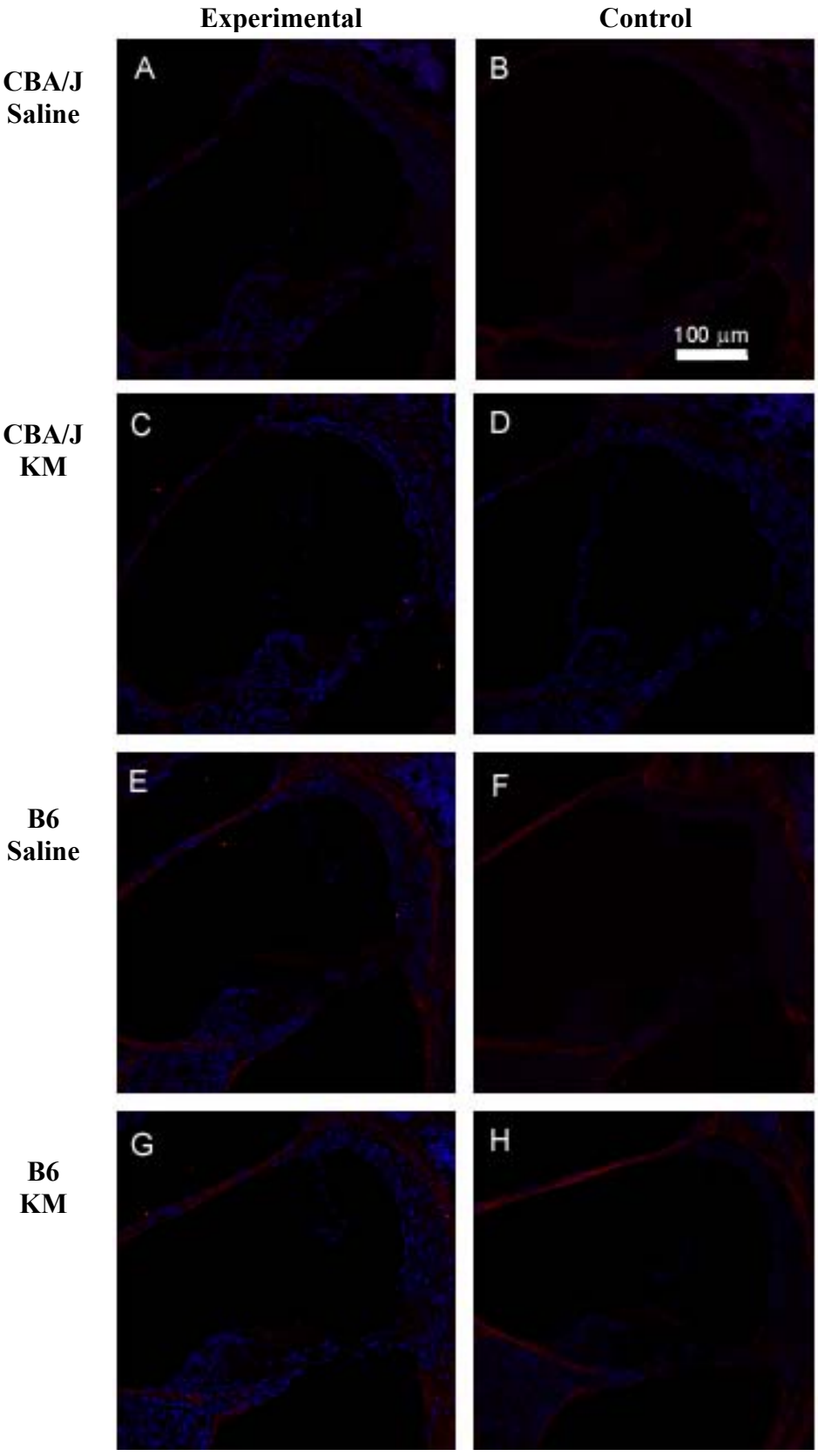

Figure 5: Cochlear upper basal turn immunolocalization of HO-1. No difference in immunoreactivity between saline and kanamycin treated B6 and CBA/J mice was observed. **(A)** Saline treated CBA/J **(B)** Saline treated CBA/J control with secondary antibody only **(C)** Kanamycin treated CBA/J **(D)** Kanamycin treated CBA/J control with secondary antibody only **(E)** Saline treated B6 **(F)** Saline treated B6 control with secondary antibody only **(G)** Kanamycin treated B6 **(H)** Kanamycin treated B6 control with secondary antibody only.

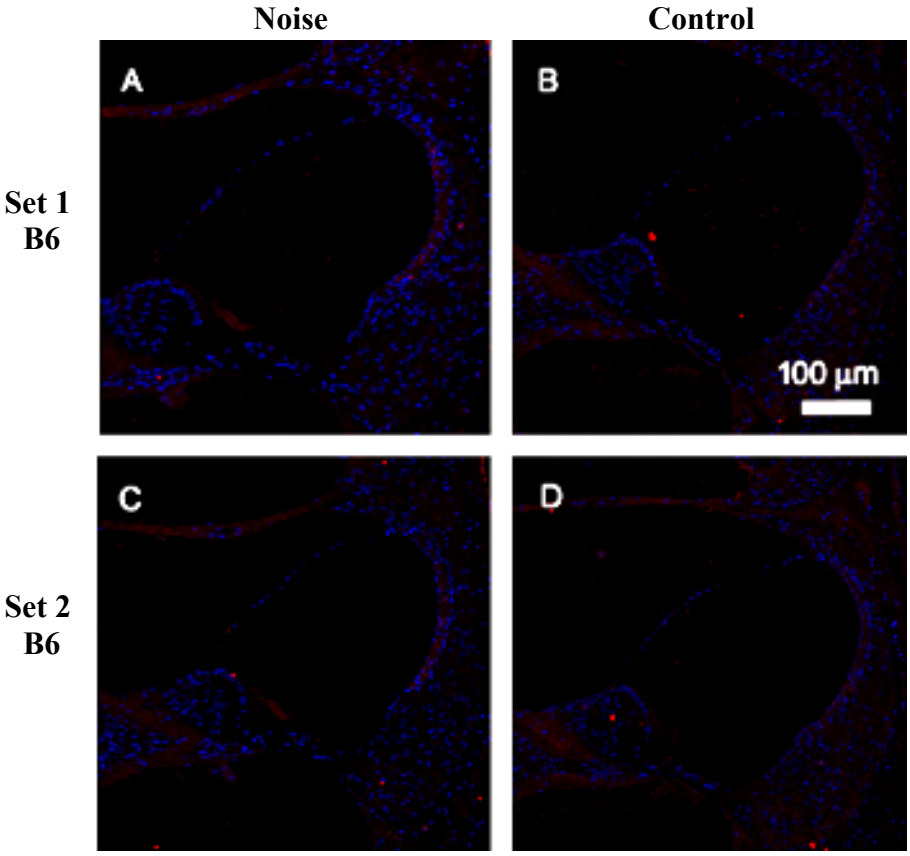

Figure 6: Cochlear HO-1 positive control using noise exposure **(A & C)**. Noise exposed B6 tissue **(B & D)** Control (unexposed) B6 tissue. In 4 out of 4 cases, increased expression of HO-1 was seen in the stria vascularis in noise exposed animals compared to 4 unexposed animals. Two of those cases are shown as Set 1 and Set 2.

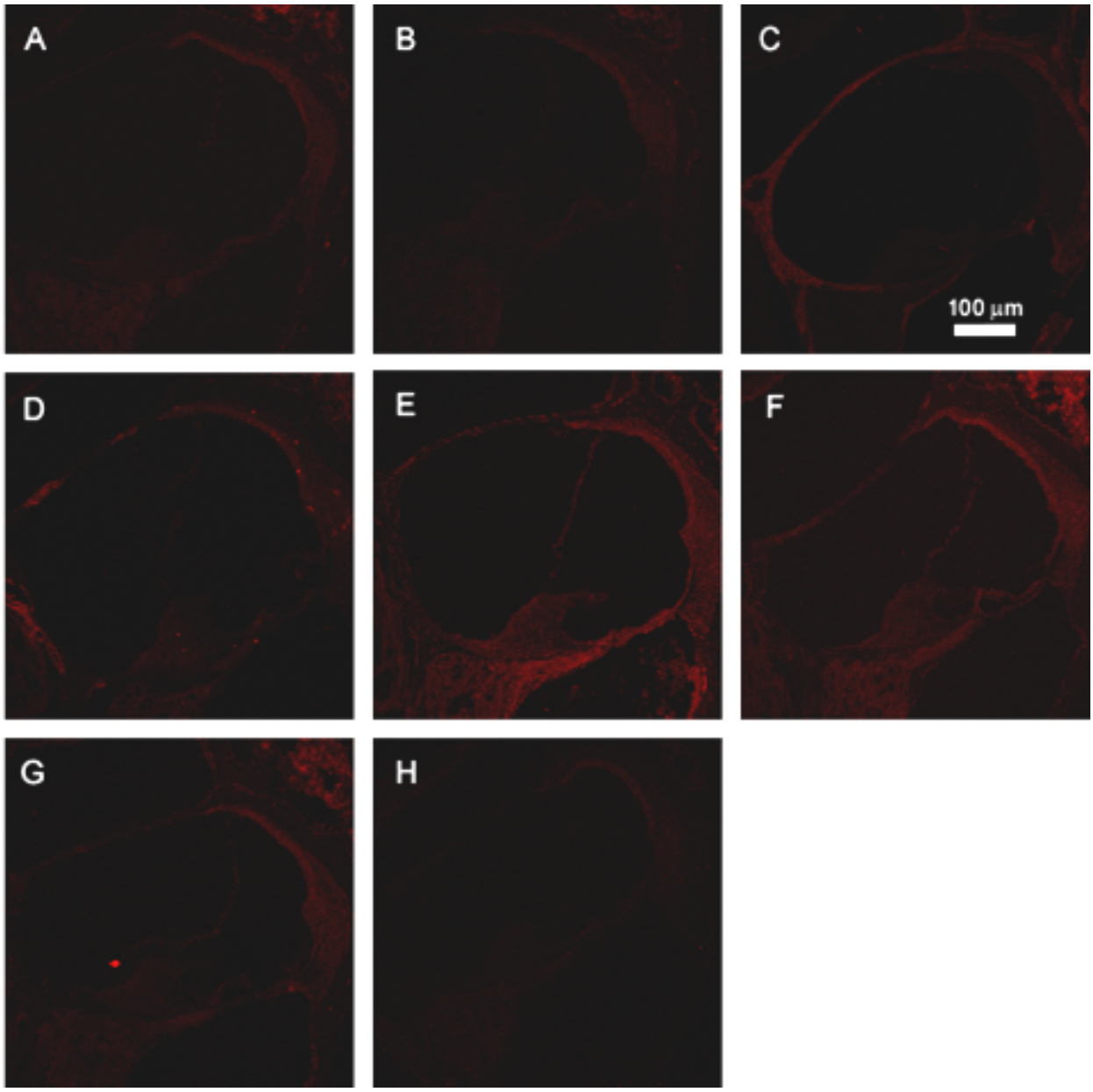

Figure 7: Cochlear upper basal turn immunolocalization of TNF-α in saline treated CBA/J mice. TNF-α was not reliably detected in CBA/J saline treated mice. **(A-G)** Incubated with 1:100 TNF-α primary and 1:1000 secondary. **(H)** Control incubated with secondary only (primary replaced with PBS). Similar immunoreactivity was noted between B6 and CBA/J saline treated mice **(Figs. 7 & 9)**.

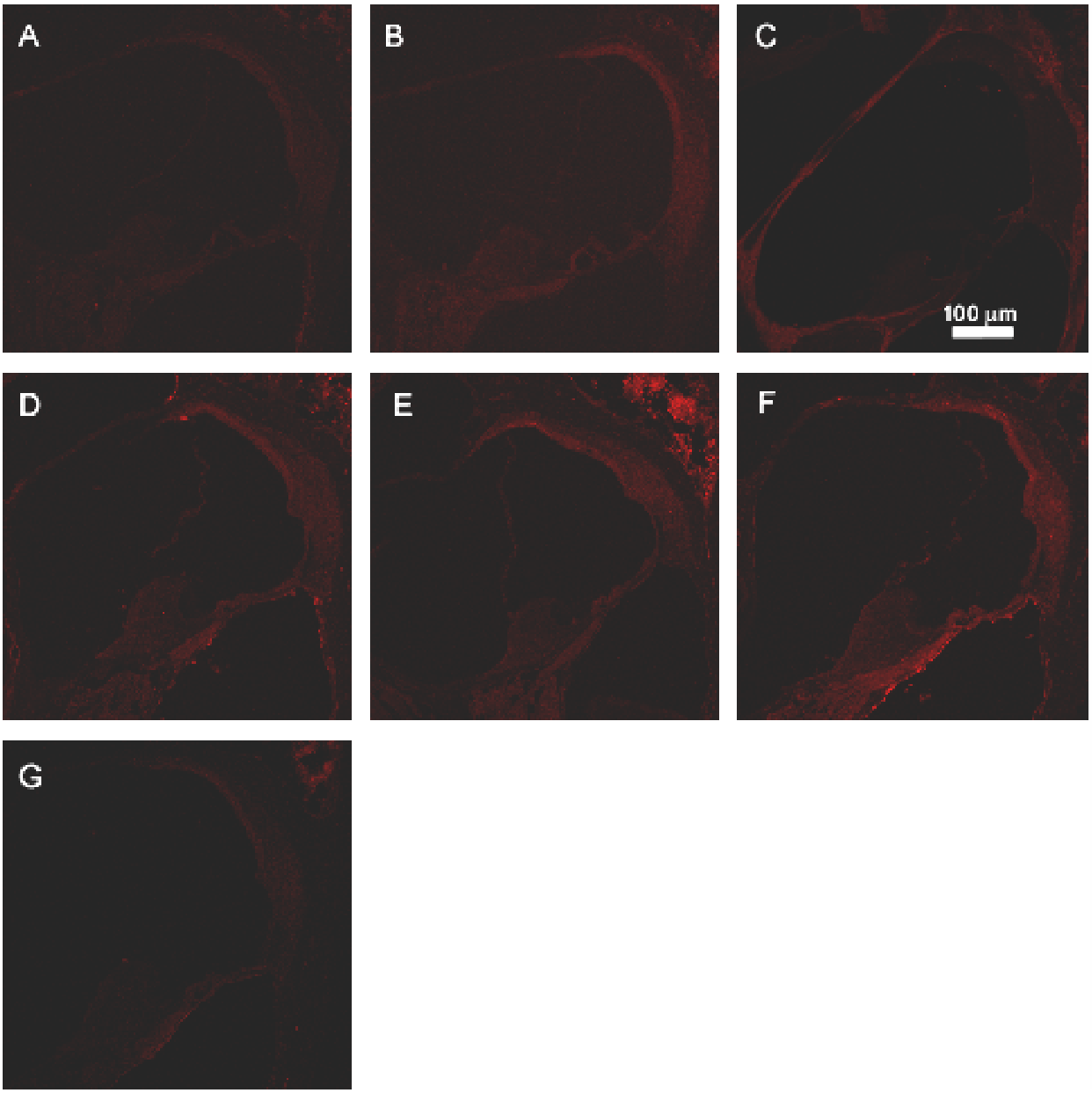

Figure 8: Immunolocalization of TNF-α in CBA/J kanamycin treated mice. Cochlear upper basal turn images. **(A-F)** Incubated with 1:100 TNF-α primary and 1:1000 secondary. **(G)** Control incubated with secondary only (primary replaced with PBS). Immunoreactivity was not reliably observed.

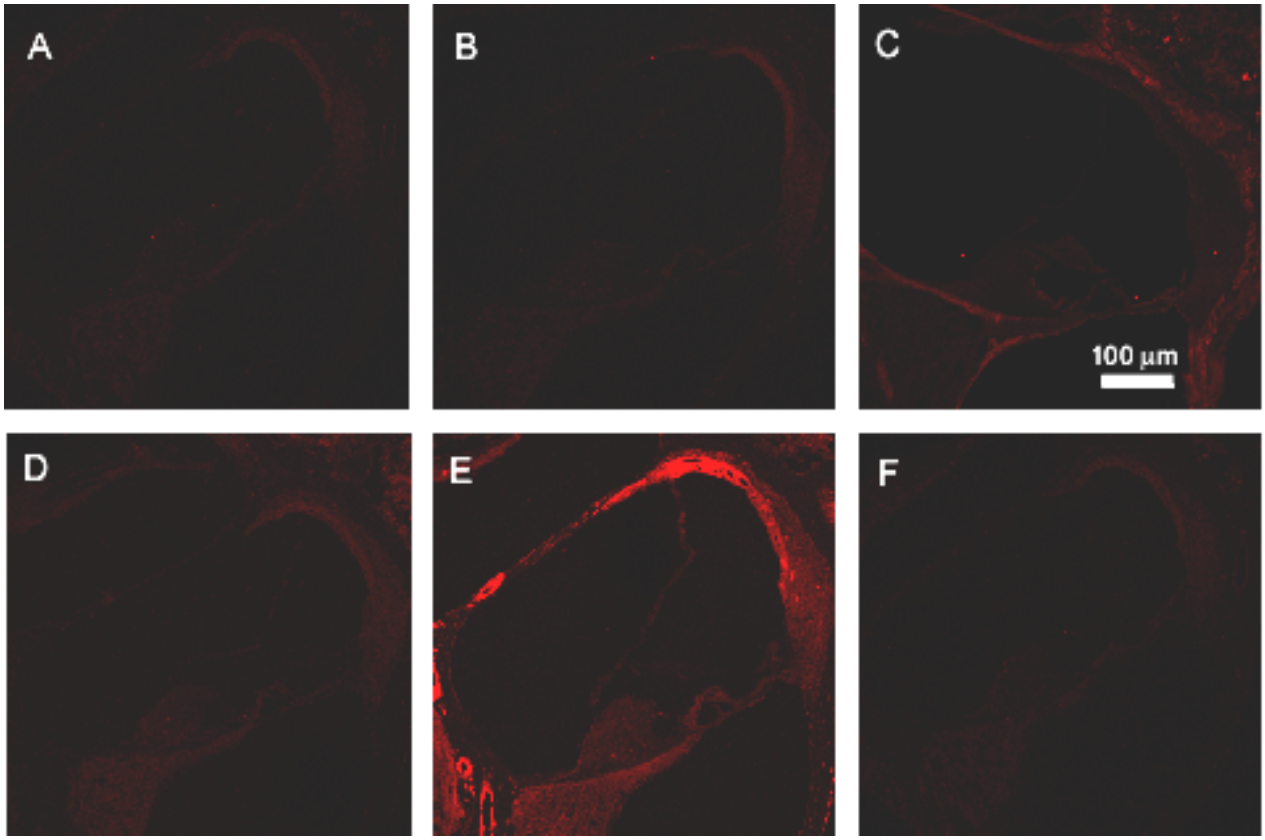

Figure 9: Immunocytochemistry suggested no reliable expression of TNF-α in B6 saline treated mice. Cochlear upper basal turn images. **(A-E)** Incubated with 1:100 TNF-α primary and 1:1000 secondary. **(F)** Control incubated with secondary only (primary replaced with PBS). Equal immunoreactivity was noted between B6 saline treated mice **(Fig. 9)** and CBA/J saline treated mice **(Fig. 7)**. Two animals were excluded due to poor cochlear preservation and histology issues rendering the sections unable to image or qualitatively analyze. The bright staining in **(E)** is likely due to artifact.

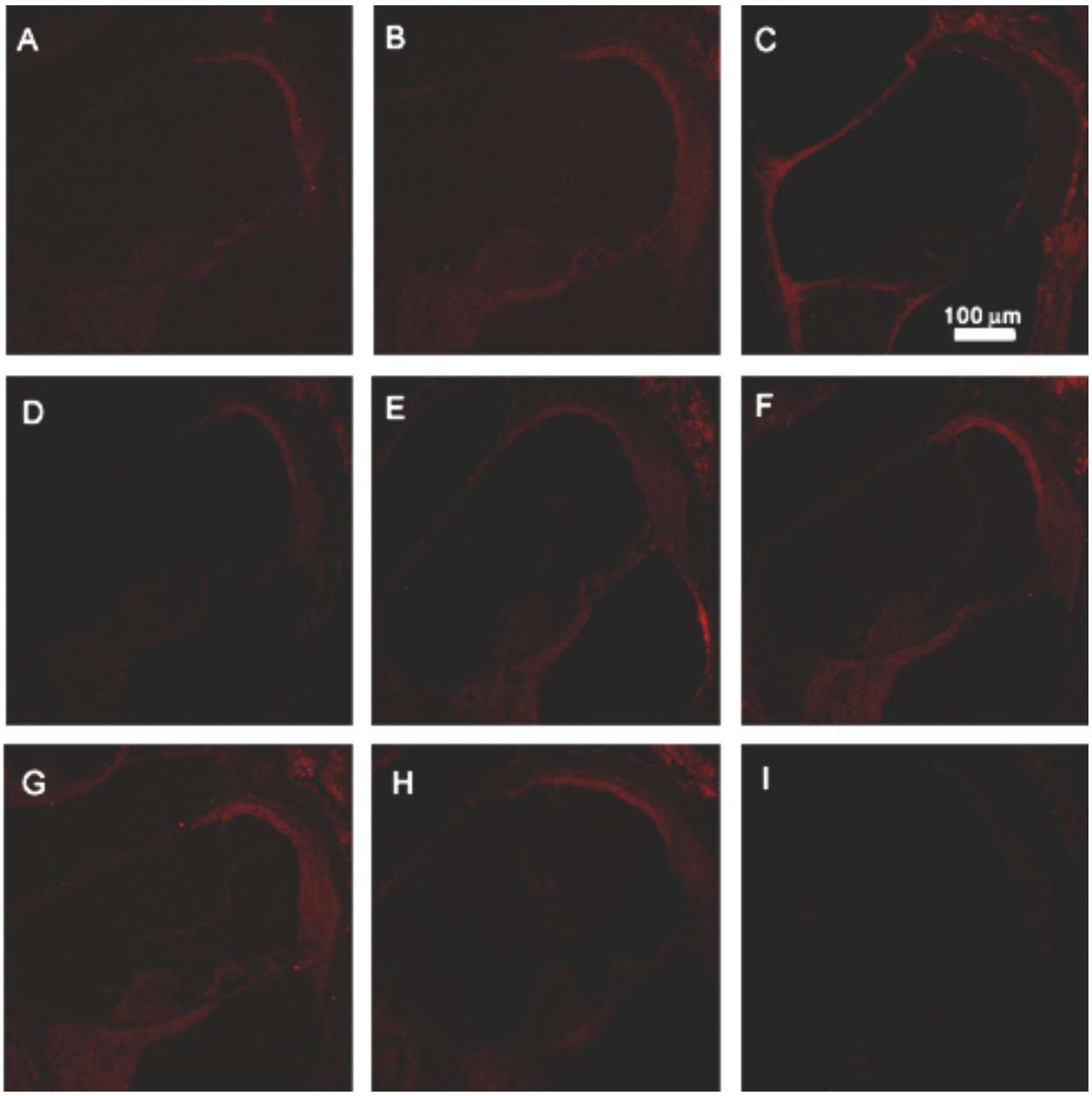

Figure 10: Immunocytochemistry for TNF-α in kanamycin treated B6 mice. In the majority of cases, there is more TNF-α expressed in the lateral wall (type II region and stria vascularis) of the B6 kanamycin treated mice **(Fig. 10)** than the CBA/J kanamycin treated mice (**Fig. 8) (A-H)** Incubated with 1:100 TNF-α primary and 1:1000 secondary. **(I)** Control incubated with secondary only (primary replaced with PBS).

DISCUSSION

The present study investigated cochlear expression of the proteins HSP70, HSF-1, HO-1 and TNF-α in kanamycin and saline treated B6 and CBA/J mice to explore the biochemical bases of kanamycin preconditioning. Since protection is found in CBA/J mice, but not found in B6 mice, differences in protein expression were examined to reveal possible allelic differences in key genes that regulate protective pathways. These may be differences either within the genes whose products were examined, or in upstream regulatory genes. We hypothesized that any of the proteins that showed greater upregulation following kanamycin in CBA/J mice than in B6 were likely to be critical to the manifestation of preconditioning. Conversely, we hypothesized that any of these showing greater upregulation in B6 mice than in CBA/J mice, especially TNF-α, were likely involved in the inhibition of preconditioning.

HSP70

Our examination revealed no expression of HSP70 in the cochlea following kanamycin treatment, raising the possibility that HSP70 does not critically mediate KM preconditioning in CBA/J. No difference in immunoreactivity was seen between saline and kanamycin treated B6 and CBA/J mice; although, we cannot be sure that HSP70 was in fact critically upregulated, but to undetectable levels. Although this finding diverges from previous studies showing HSP70 expression in mouse and rat cochlea following cisplatin, heat shock, neomycin, and ischemia (Myers et al., 1992; Oh et al., 2000; Cunningham & Brandon, 2006; Taleb et al., 2008; Taleb et al., 2009; Garcia-Berrocal et al., 2010), the effects of low-dose KM have not been tested.

HO-1

Our observations revealed no expression of HO-1 as a function of strain (B6 vs. CBA/J)

or treatment (kanamycin vs. saline), suggesting that expression of HO-1 does not critically mediate KM preconditioning in CBA/J mice. Positive controls using noise exposure suggested that HO-1 is expressed in noise treated cochlear tissue at detectable levels. HO-1 expression has been shown after heat shock in the rat cochlea (Fairfield et al., 2004), but any changes due to low-dose KM have not been tested. Our finding of HO-1 in stria vascularis stands in contrast to observations in the guinea pig cochlea, where staining was found in outer hair cells, and not the stria vascularis following noise exposure (Matsunobu et al., 2009).

HSF-1

HSF-1 findings of the present study were consistent with previous studies. Expression of HSF-1 in the organ of Corti, interdental cells of spiral limbus, and inner sulcus cells was observed; however, no difference in HSF-1 immunoreactivity was indicated by strain (B6 or CBA/J) or treatment (kanamycin vs. saline). This suggested that HSF-1 was present in equally detectable quantities in all conditions, in agreement with previous literature (Higashi et al., 1995), yet does not critically mediate KM preconditioning in CBA/J mice. Fairfield et al. (2002) showed expression of HSF-1 in the inner and outer hair cells, spiral ganglion cells and stria vascularis of the rat and mouse cochlea. Findings from the present study overlapped with regard to expression in the inner and outer hair cells, but not in our findings of expression in the spiral limbus and inner sulcus cells. This difference may be attributed to the fact that the animals in the present study were not heat shocked, which is known to dramatically upregulate expression of heat shock proteins (Higashi et al., 1995; Fairfield et al., 2002; Taleb et al., 2008). Present and previous findings of HSF-1 under normal conditions are expected, given that it is constitutively expressed, and becomes transcriptionally active under stressful conditioning (Pirkkala et al.,

2001).

When our results with HSP70, HO-1, and HSF-1 are taken together, there is a consistency, since HSF-1 is the single most prominent 'driver' of HSP70 and HO-1 transcription. Thus, if KM preconditioning in CBA/J mice does not critically involve HSPs, these results should all correspond, as we observed them to. Although HSPs are widely activated under stress, and as part of preconditioning cascades, not all preconditioning need involve HSPs. For example, no prominent role for HSPs has been identified in protection against noise injury conferred by restraint (Wang & Liberman, 2002).

TNF-α

The present study revealed TNF-α expression in B6 kanamycin treated mice compared to CBA/J kanamycin treated mice, specifically in the lateral wall, suggesting that expression of TNF-α varies with genetic background. Overall, no immunoreactivity was observed in saline or kanamycin treated CBA/J or saline treated B6 mice. TNF-α expression has previously been found in the lateral wall, along with the reticular lamina, organ of Corti, spiral ligament, spiral vascular prominence and cochlear vasculature, and spiral ganglion (Hess et al., 1999; Zou et al., 2005). Due to the potential role of TNF-α in promoting cell damage and protection against apoptosis, upregulation of TNF-α in B6 mice could contribute to the subtle injury of B6 cochlea by KM suggested by earlier experiments. This tipping of the balance toward injury by inflammatory processes that involve TNF-α may represent the primary factor in why CBA/J mice favor protection by KM, while B6 mice seem to favor injury.

CONCLUSIONS

The present findings indicate that neither HSP70 nor HO-1 are critical mediators of kanamycin preconditioning in CBA/J mice. It cannot be ruled out, of course, that sub-detectable upregulation of HSP70 or HO-1 playing an important role in preconditioning was simply missed. The apparent presence of HSF-1 under all conditions, combined with the apparent non-upregulation of the HSPs we examined, paint an internally consistent picture in arguing that KM preconditioning in CBA/J does not require these HSPs. In contrast, the indicated upregulation in the cochlea of TNF-α by kanamycin only in B6 mice suggests that differences in TNF-α upregulation, an upstream process, or perhaps inflammatory responses to low-dose kanamycin in this strain are a major contributor to the apparent net harmful effects of kanamycin in B6. Ultimately, gene expression experiments may be required to resolve these issues. The apparent 'non-protectability' of B6 by kanamycin may point to genes that modulate the risk of hearing loss due to environment in animals and humans.

REFERENCES

Canlon, B., Henderson, D., & Salvi, R. (2007). Pharmacological strategies for prevention and treatment of hearing loss and tinnitus. *Hearing Research, 226,* 1-2.

Cunningham, L. L., & Brandon, C. S. (2006). Heat shock inhibits both aminoglycoside- and cisplatin-induced sensory hair cell death. *Journal of the Association for Research in Otolaryngology, 7,* 299-307.

Darrat, I., Ahmad, N., Seidman, K., & Seidman, M. D. (2007). Auditory research involving antioxidants. *Current Opinions in Otolaryngology and Head and Neck Surgery, 15*, 358-363.

Das, D. K., & Maulik, N. (2003). Preconditioning potentiates redox signaling and converts death signal into survival signal. *Archives of Biochemistry and Biophysics, 420*, 305-311.

Dirnagl, U., Simon, R. P., & Hallenbeck, J. M. (2003). Ischemic tolerance and endogenous neuroprotection. *Trends in Neuroscience, 26*, 248-254.

Eisen, A., Fisman, E. Z., Rubenfire, M., Freimark, D., McKechnie, R., Tenenbaum, A., Motro, M., & Adler, Y. (2004). Ischemic preconditioning: nearly two decades of research. A comprehensive review. *Atherosclerosis, 172*, 201-210.

Fairfield, D. A., Kanicki, A. C., Lomax, M. I., & Altschuler, R. A. (2002). Expression and localization of heat shock factor (Hsf) 1 in the rodent cochlea. *Hearing Research, 173,* 109-118.

Fairfield, D. A., Kanicki, A. C., Lomax, M. I., & Altschuler, R. A. (2004). Induction of heat shock protein 32 (Hsp32) in the rat cochlea following hyperthermia. *Hearing Research, 188,* 1-11.

Fernandez, E. A., Ohlemiller, K. K., Gagnon, P. M., & Clark, W. W. (2010). Protection against noise-induced hearing loss in young CBA/J mice by low-dose kanamycin. *Journal of the Association for Research in Otolaryngology, 11,* 235-244.

Gagnon, P. M., Simmons, D. D., Bao, J., Lei, D., Ortmann, A. J., & Ohlemiller, K. K. (2007). Temporal and genetic influences on protection against noise-induced hearing loss by hypoxic preconditioning in mice. *Hearing Research, 226,* 79-91.

Garcia-Berrocal, J. R., Nevado, J., Gonzalez-Garcia, J. A., Sanchez-Rodriguez, C., Sanz, R., Trinidad, A., Espana, P., Citores, M. J., & Ramirez-Camacho, R. (2010). Heat shock protein 70 and cellular disturbances in cochlear cisplatin ototoxicity model. *The Journal of Laryngology and Otology, 124,* 599-609.

Gidday, J. M. (2006). Cerebral preconditioning and ischaemic tolerance. *Nature Reviews Neuroscience, 7*, 437-448.

Goodson, M. L., & Sarge, K. D. (1995). Regulated expression of heat shock factor 1 isoforms with distinct leucine zipper arrays via tissue-dependent alternative splicing. *Biochemical and Biophysical Research Communcations, 211*(3), 943-949.

Hess, A., Bloch, W., Huverstuhl, J., Su, J., Stennert, E., Addicks, K., & Michel, O. (1999). Expression of inducible nitric oxide synthase (iNOS/NOS II) in the cochlea of guinea pigs after intratympanical endotoxin-treatment. *Brain Research, 830,* 113-122.

Higashi, T., Nakai, A., Uemura, Y., Kikuchi, H., & Nagata, K. (1995). Activation of heat shock factor 1 in rat brain during cerebral ischemia or after heat shock. *Molecular Brain Research, 34, 262-270.*

Kawamoto, K., Sha, S. H., Minoda, R., Izumikawa, M., Kuriyama, H., Schacht, J., & Raphael, Y. (2004). Antioxidant gene therapy can protect hearing and hair cells from ototoxicity. *Molecular Therapy, 9*(2), 173-181.

Le, T., & Keithley, E. M. (2007). Effects of antioxidants on the aging inner ear. *Hearing Research, 226,* 194-202.

Le Prell, C. G., Yamashita, D., Minami, S. B., Yamasoba, T., & Miller, J. M. (2007). Mechanisms of noise-induced hearing loss indicate multiple methods of prevention. *Hearing Research, 226,* 22-43.

Matsunobu, T., Satoh, Y., Ogawa, K., & Shiotani, A. (2009). Heme oxygenase-1 expression in the guinea pig cochlea induced by intense noise stimulation. *Acta Oto-Laryngologica, 129*(562), 18-23.

Matz, G. (1993). Aminoglycoside cochlear ototoxicity. *Otolaryngologic Clinics of North America, 26*, 705-712.

Myers, M. W., Quirk, W. S., Rizk, S. S., Miller, J. M., & Altschuler, R. A. (1992). Expression of the major mammalian stress protein in the rat cochlea following transient ischemia. *Laryngoscope, 102,* 981-987.

Niu, X., Shao, R., & Canlon, B. (2003). Suppression of apoptosis occurs in the cochlea by sound conditioning. *NeuroReport, 14*(7), 1025-1029.

Oh, S. H., Yu, W. S., Song, B., Lim, D., Koo, J., Chang, S. O., & Kim, C. (2000). Expression of heat shock protein 72 in rat cochlea with cisplatin-induced acute ototoxicity. *Acta Otolaryngology, 120,* 146-150.

Ohlemiller, K. (2006). Contributions of mouse models to understanding of age- and noise-related hearing loss. *Brain Research, 1091,* 89-102.

Ohlemiller, K.K., Rybak Rice, M. E., Rosen, A. D., Montgomery, S. C., & Gagnon, P. M. (In Press). Protection by low-dose kanamycin against noise-induced hearing loss in mice: dependent on dosing regimen and genetic background. *Hearing Research*.

Pasupathy, S., & Homer-Vanniaksinkam, S., (2005). Ischaemic preconditioning protects against ischaemia/reperfusion injury: emerging concepts. *European Journal of Vascular and Endovascular Surgery, 29*, 106-115.

Perletti, G., Vral, A., Patrosso, M. C., Marras, E., Ceriani, I., Willems, P., Fasano, M., & Magri, V. (2008). Prevention and modulation of aminoglycoside ototoxicity (Review). *Molecular Medicine Reports, 1*, 3-13.

Pirkkala, L., Nykanen, P., & Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *The FASEB Journal, 15,* 1118-1131.

Prass, K., Scharff, A., Ruscher, K., Lowl, D., Musselmann, C., Victorov, I., Kapinya, K., Dirnagl, U., & Meisel, A. (2003). Hypoxia-induced stroke tolerance in the mouse in mediated by erythropoietin. *Stroke, 34*, 1981-1986.

Priuska, E. M., & Schacht, J. (1997). Mechanism and prevention of aminoglycoside ototoxicity: outer hair cells as targets and tools. *Ear Nose & Throat Journal, 76*, 164-171.

Ran, R., Xu, H., Lu, A., Bernaudin, M., & Sharp, F. R. (2005). Hypoxic preconditioning in the brain. *Developmental Neuroscience, 27*, 87-92.

Rice, Mary Elizabeth Rybak, "Optimal dose of Kanamycin for protection from noise induced hearing loss" (2009). *Independent Studies and Capstones.* Paper 311. Program in Audiology and Communication Sciences, Washington University School of Medicine. http://digitalcommons.wustl.edu/pacs_capstones/311

Rizzi, M. D., & Hirose, K. (2007). Aminoglycoside ototoxicity. *Current Opinion in Otolaryngology & Head and Neck Surgery, 15,* 352-357.

Rosen, Allyson D., "Genetic and temporal aspects of protection by kanamycin against cochlear noise injury" (2010). *Independent Studies and Capstones.* Paper 597. Program in Audiology and Communication Sciences, Washington University School of Medicine. http://digitalcommons.wustl.edu/pacs_capstones/597

Satoh, H., Firestein, G. S., Billings, P. B., Harris, J. P., & Keithley, E. M. (2002). Tumor necrosis factor-α, an initiator, and etanercept, an inhibitor of cochlear inflammation. *The Laryngoscope, 112,* 1627-1634.

Schacht, J. (1993). Biochemical basis of aminoglycoside ototoxicity. *Otolaryngologic Clinics of North America, 26,* 845-856.

Schacht, J. (1999). Antioxidant therapy attenuates aminoglycoside-induced hearing loss. *Annals of the New York Academy of Sciences, 884*, 125-130.

Scherer, E. Q., Yang, J., Canis, M., Reimann, K., Ivanov, K., Diehl, C. D., Backx, P.H., Wier, W. G., Strieth, S., Wangemann, P., Voigtlaender-Bolz, J., Lidington, D., & Bolz, S. S. (2010). Tumor necrosis factor – {alpha} enhances microvascular tone and reduces blood flow in the cochlea via enhanced sphingosine-1-phosphate signaling. *Stroke, 41,* 2618-2624.

Seidman, M. D., & Vivek, P. (2004). Intratympanic treatment of hearing loss with novel and traditional agents. *Otolaryngologic Clinics of North America, 37*(5), 973-990.

Shen, H., Zhange, B., Shin, J. H., Lei, D., Du, Y., Gao, X., Dai, C., Ohlemiller, K. K., & Bao, J. (2006). Prophylactic and therapeutic functions of T-type calcium blockers against noise-induced hearing loss. *Hearing Research, 226*, 52-60.

Sommers-child, H. T., & Kirkeboen, K. A. (2002). Preconditioning-endogenous defense mechanisms of the heart. *Acta Anesthesiologica Scandinavica, 46*, 123-137.

Sugahara, K., Inouye, S., Izu, H., Katoh, Y., Katsuki, K., Takemoto, T., Shimogori, H., Yamashita, H., & Nakai, A. (2003). Heat shock transcription factor HSF1 is required for survival of sensory hair cells against acoustic overexposure. *Hearing Research, 182,* 88-96.

Taleb, M., Brandon, C. S., Lee, F., Lomax, M. I., Dillmann, W. H., & Cunningham, L. (2008). Hsp70 inhibits aminoglycoside-induced hair cell death and is necessary for the protective effect of heat shock. *Journal of the Association for Research in Otolaryngology, 9,* 277-289.

Taleb, M., Brandon, C. S., Lee, F., Harris, K. C., Dillmann, W. H., & Cunningham, L. L. (2009). Hsp70 inhibits aminoglycoside-induced hearing loss and cochlear hair cell death. *Cell Stress and Chaperones, 14,* 427-437.

Tran Ba Huy, P., Bernard, P., & Schacht, J. (1986). Kinetics of gentamicin uptake and release in the rat: comparison of inner ear tissues and fluids with other organs. *Journal of Clinical Investigations, 77,* 1492-1500.

Van De Water, T. R., Lallemend, F., Eshraghi, A. A., Ahsan, S., He, J., Guzman, J., Polak, M., Malgrange, B., Lefebvre, P. P., Staecker, H., & Balkany, T. J. (2004). Caspases, the enemy within, and their role in oxidative stress-induced apoptosis of inner ear sensory cells. *Otology & Neurotology, 25*(4), 627-632.

Wang, Y., & Liberman, M. C. (2002). Restraint stress and protection from acoustic injury in mice. *Hearing Research, 165,* 96-102.

Watanabe, K., Oshima, T., Kobayashi, T., & Ikeda, K. (2003). The expression and localization of heme oxygenase in the adult guinea pig cochlea. *Brain Research, 966,* 162-166.

Wu, W., Sha, S., McLaren, J. D., Kawamoto, K., Raphael, Y., & Schacht, J. (2001). Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. *Hearing Research, 158,* 165-178.

Yoshida, N., Kristiansen, A., & Liberman, M. C. (1999). Heat stress and protection from permanent acoustic injury in mice. *The Journal of Neuroscience, 19*(22), 10116-10124.

Yoshida, N., & Liberman, M. C. (2000). Sound conditioning reduces noise-induced permanent threshold shift in mice. *Hearing Research, 148,* 213-219.

Yu, L., Jiang, X. H., Zhou, Z., Tsang, L. L., Yu, M. K., Chung, Y. W., Zhang, X. H., Wang, A. M., Tang, H., & Chan, H. C. (2011). A protective mechanisms against antibiotic-induced ototoxicity: role of prestin. *PLoS one, 6*(2), 1-8.

Zhao, J., Brooks, D. M., & Lurie, D. I. (2006). Lipopolysaccharide-activated SHP-1-deficient motheaten microglia release increased nitric oxide, TNFα, and IL-1β. *GLIA, 53,* 304-312.

Zou, J., Pyykko, I., Sutinen, P., & Toppila, E. (2005). Vibration induced hearing loss in guinea pig cochlea: expression of TNFα and VEGF. *Hearing Research, 202,* 13-20.

Appendix A: Injection Sheet

Date: AM

Ear notch	Animal ID	Treatment	BW (g) (x 0.006)	Injection Volume (µL)
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				

Date: PM

Ear notch	Animal ID	Treatment	BW (g) (x 0.006)	Injection Volume (µL)
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				
( )				