Biochemical and Bioenergetic Alterations in Cochlear Tissue: A Comparative Study in Wild Type and Alport Syndrome Knockout Mice Under Quiet and Noise Conditions

Brendan Smyth[1], Jared Hartsock[2], Nathan Yates Nelson[1], Linda Weisenmiller[1], Dominic Cosgrove[1], Michael Anne Gratton[1]

[1]Boys Town National Research Hospital, Omaha, NE, [2]Turner Scientific, Jacksonville, Ill.

Introduction

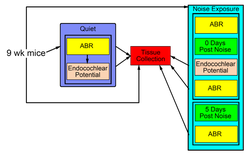
Study of bioenergetic and biochemical alterations contributing to cochlea have been constrained by the limited tissue sample and the sensitivity/resolution of analytical methods. Advancements in high-resolution measurement technology and enhanced assay sensitivity should allow for more precise and detailed analysis of bioenergetic changes in single mouse cochlear tissues. We hypothesize that noise exposure amplifies existing metabolic imbalances, potentially implicating mitochondrial dysfunction culminating in accelerated cochlear impairment. Furthermore, we query the ability of current technology with use of single mouse cochlear samples.

Objective

To assess the bioenergetic and biochemical alterations in cochlear tissues of 129 Sv wild type and Alport Syndrome mice under quiet and noise-induced metabolic stress conditions.

Methods

Study Design



- 129Sv Wild type (WT) and Collagen 4α3 (Alport) KO mice, aged 9 weeks of both sex were used.
- At 9 wks, with the exception of quiet WT and KO groups, mice were exposed to a metabolic noise stressor (10kHz OBN,106 dB SPL,10H) Subsets of each group of quiet or metabolically noise-stressed mice underwent ABR testing. The metabolically stressed mice were evaluated both prior to and at 0- or 5-days post -metabolic stressor. In a 2nd subset, the endocochlear potential (EP) was measured in quiet-reared mice or 0 days post-noise.
- in a 3rd subset of WT and KO quiet-reared mice, measures of the strial capillary basement membrane width were obtained from electron micrographs. (Meehan et al, 2016).

Data Collection (n=5-10/group)
Data were collected from quiet-reared mice and from metabolically stressed mice at two post-noise time points: immediately post-exposure (0D) and five (5D) days post-exposure.

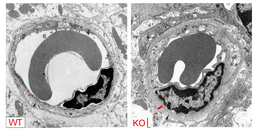
- Hearing at 9 wks was assessed via the ABR technique with frequency-specific tone pips (8,16,24,32 & 40 kHz) (Meehan et al., 2016).
- The DC voltage of the EP was measured using a 150 mM KCl-filled glass pipette (approx. 1.1 um tip via a retro-auricular approach to access the cochlear basal turn.
- The 3rd subset of mice was transcardiac perfused, cochlea were prepared for transmission electron microscopy. The thickness of strial capillary basement membranes was measured in TEM digital images taken at 40,000x.
- With the exception of the 3rd subset of mice, the unfixed stria vascularis (SV) or the organ of Corti (OC) apical turn was microdissected (HBSS, pH 7.4, 4°C), quick frozen and stored (-80°C) until assay (Gratton et al., 2005).
- For all bioenergetic assays, the isolated tissue were thawed on ice, resuspended in 30µl of homogenizing solution, homogenized and centrifuged (4°C, 10g, 15min) to obtain pellet and supernatant (Gratton et al., 1995, 1997). The supernatant was divided in half and diluted with the respective lysis buffers. Measurements were referenced to total protein Pierce™ BCA Protein assay, Thermo Scientific). Colored or clear low volume 384 or half area 96 well microplates (Corning) on a Molecular Diagnostics SpectraMax iD5 reader were utilized.

Data analysis
- Hearing loss equalled the decibel difference between the pre- and post- noise thresholds. The threshold shift was subjected to 2-way ANOVA with post-hoc analysis via Holm-Sidak multiple-comparison test as were the measures of EP magnitude and strial capillary basement membrane width.
- The Kruskal-Wallis test was applied for nonparametric data. Post-hoc analysis consisted of the Mann-Whitney U test with Bonferroni correction for pairwise comparsion.

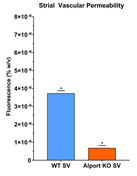
For all statistical analyses, significance was set at p<0.05. For figures: *p<0.05, **p<0.01, ***p<0.001.

Alport KO SV in Metabolic Stress

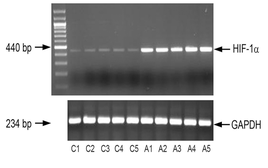
Col4a3 mutations cause a progressive accumulation of select extracellular matrix molecules in strial capillary basement membranes, resulting in decreased vascular permeability leading to the presence of cochlear hypoxia. Ultimately, functional defects include strial dyshomeostasis, a lower EP and susceptibility to metabolic stress.



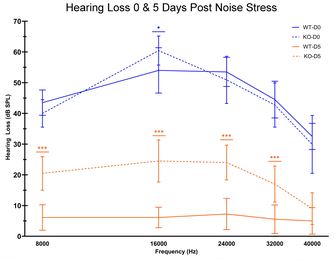
Alport KO mice have thickened Strial Capillary Basement Membranes.
- Red bars on the electron micrographs illustrate capillary basement membrane width. The mean width value in the KO SV capillaries was 114.6 nm, almost double that of the WT value of 54.8 nm. Importantly, the strial capillary basement membrane is the sole cochlear site that is significantly thickened (2-way ANOVA, post-hoc Holm-Sidak p<0.05).



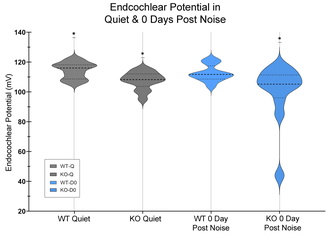
Thick capillary basement membranes reduce SV vascular permeability. The KO SV fluorescence is significantly lower than that in WT SV following 3 min incubation of rhodamine dye injected into the left ventricle followed by PBS perfusion of the vasculature. ANOVA p<0.001 (Dufek et al., 2020).



Upregulation of HIF-1a mRNA in KO SV. The presence of a local hypoxic environment is indicated by the 5-fold increase in HIF-1α mRNA in the Alport SV.

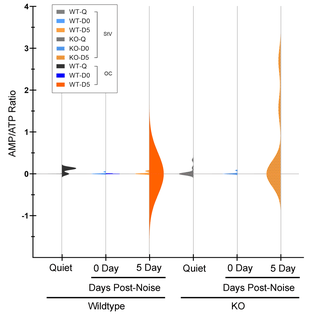


KO mice exhibit mild hearing loss after metabolically stressful noise. WT mice show near complete recovery of hearing loss following exposure to metabolic stress (10 kHz OBN, 106 dB SPL, 10H). KO mice have incomplete recovery. Pre-noise stress hearing was comparable between groups, with the exception of a mild high frequence loss in KO. Data are shown as MEAN±SEM.

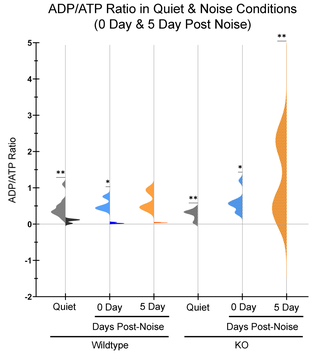


Endocochlear potential decreases after noise exposure in Alport KO mice.
- The EP values in the quiet-reared KO mice were significantly lower than those of quiet-reared WT mice (ANOVA, p<0.05). At 0 days post noise, the EP minimally changed in WT mice, but dropped significantly in the KO mice to a mean of 100 mV, reflecting the wider range of the EP magnitude.

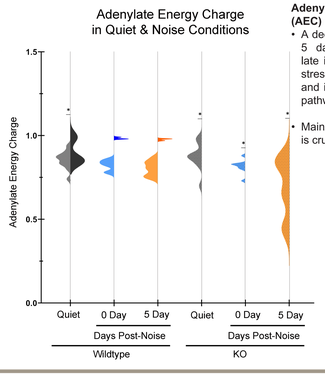
Results



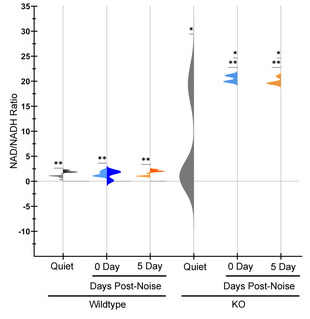
AMP/ATP Ratio Before and After Noise:
- Stable AMP/ATP ratios across conditions suggest metabolic equilibrium.
- However, in KO mice 5 days post noise exposure, a notable split into high and low AMP/ATP ratios indicates disrupted energy balance post-noise exposure for some mice in this group. The higher ratios imply that AMPK should be activated. This is consistent with maladaptation to energy sensing.
- The AMP/ATP ratio distribution at D5 post-noise is unexpected and warrants further investigation.



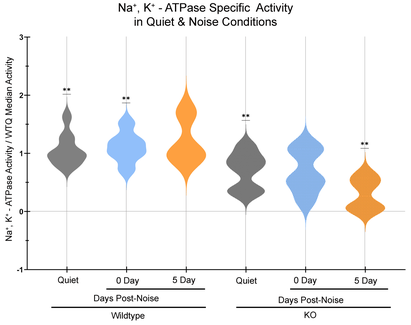
ADP/ATP Ratio Indicates Energy Status:
- Significant differences in ADP/ATP ratios post-noise reveal shifts from ATP production to consumption, suggesting oxidative stress and potential mitochondrial dysfunction.
- Similar to elevated AMP/ATP ratios, the elevated ADP/ATP ratio in the KO noise conditions should indicate AMPK activation.
- The significantly increased range in the ADP/ATP distribution of KO 5D indicates loss of energy balance and maladaptation to energy sensing.



Adenylate Energy Charge (AEC) and Tissue Survival:
- A decreased AEC in KO mice 5 days post-exposure is a late indicator of mitochondrial stress, tissue senescence and initiation of the cell death pathway.
- Maintaining AEC at a 0.8 ratio is crucial for tissue survival.



Maladaptive Differences in the KO NAD/NADH Ratio Indicate an Abnormal Redox State:
- In the quiet KO condition, some KO mice have a greater instability in their redox state related to higher oxidative stress vs KO mice whose NAD/NADH ratio approximates that of the WT mice.
- Elevated NAD/NADH ratios in KO noise conditions imply increased oxygen demand yet the KO SV is in a hypoxic state potentially indicating a shift towards glycolysis and a change in energy substrate preference.



Significant Differences in WT vs KO Na^+,K^+-ATPase enzymatic activity
- Na^+,K^+-ATPase enzymatic activity differs between the WT and KO mice particularly in response to a metabolic noise stressor.
- Decreased Na^+,K^+-ATPase activity in KO mice post-noise could stem from limited ATP availability or altered priorities for ATP use under stress.
- Mitochondrial changes inferred from NAD/NADH ratios may also indirectly affect Na^+,K^+-ATPase enzymatic activity.

Preliminary Comparative Analysis of Exploratory Stress Biomarkers

Tissue	Condition	pAMPK/ Total AMPK	p21 pg/µg Protein	p53/Total p53	mTOR/ Total mTOR	Cytochrome C pg/ µg protein
SV	WTQ	N	N	N	N	N
SV	WTN0	↓	↓	N	N	↑
SV	WTN5	↓R	↓	N	N	↑R
SV	KOQ	<N	<N	>N	<N	>N
SV	KON0	↓	↓	N	N	↓
SV	KON5	↓	↓	↓	↓R	↑
O of C	WTQ	N	N	N	N	N
O of C	WTN0	↓	↓	N	↑	↓
O of C	WTN5	↓R	↓	↓	↑	↓R

'N' indicates levels approximately the WTQ SV levels; arrows indicate a decrease or increase compared to N; R denotes signs of recovery.

ELISA Preliminary Results Suggest an Intergraterd Stress Response
- Metabolic Stress Markers (pAMPK, mTOR, Cytochrome C) denote a change in energy sensing and mitochondrial function.
- Cell Senescence Markers (p21, p53) varied levels indicate altered cell cycle and stress response, with potential increases suggesting heightened senescence or DNA damage response.

Conclusions

Model for Cochlear Metabolic Stress:
- Data indicate significant deviations in adenylate ratios and redox states between WT and KO mice, even in the quiet conditions.
- KO mice in quiet showed a maladaptive increase in ATP/ADP ratios along with elevated Total Protein levels, fibrosis (thickened basement membranes). This implies initiation of Integrated Stress Response and changes in preferred energy substrate prior to added metabolic noise stress.
- The observed differences in biomarkers pilot data also point to mitochondrial dysfunction in the KO mice. These findings offer new insights into the cellular mechanisms underlying cochlear dysfunction.

Advancements in Tissue Analysis:
- The results demonstrate the feasibility of using single SV and OC cochlear tissues dissected from a single mouse, thanks to the sensitivity and specificity of modern assays and equipment. Certain conventional ELISAs allow transfer of samples from one assay plate to the next, thus conserving samples and allowing biomarker panels to be performed.

Future Translational Directions:
- Seahorse Assays: Utilize seahorse assays to assess mitochondrial function, metabolic flux and substrate preference in metabolically stressed cochlear tissues. These assays will provide a detailed understanding of mitochondrial respiration and glycolysis, essential for pinpointing metabolic dysfunctions in cochlear cells under stress.
- High Throughput Biomarker Assays: Implement high throughput biomarker assays for biomarkers to evaluate broader metabolic and stress responses, enhancing the understanding of complex signaling pathways involved in cochlear stress and damage.

Generalization to Other Hearing Loss Models:
- Bioenergetics analysis, including the biomarker selection and tissue analysis approach, applied to other models of hearing loss, offers a pathway for broader research into cochlear pathophysiology and potential therapeutic strategies. This opens the door to future targeted therapies.

References

Cosgrove D, Gratton MA, Madison J, Vosik D, Samuelson G, Meehan D, Delimont D, Phillips G, Smyth B, Pramparo T, Jarocki D, Nguyen M, Komers R, Jenkinson C. (2023) Dual inhibition of the endothelin and angiotensin receptor ameliorates renal and inner ear pathologies in Alport mice. J Pathol.260(3):353-364.

Dufek,B., Meehan, D., Delimont, D., Whilhelm, K, Samuelson, G., Coenen, R., Doyle E.J.,III, Smyth, B.S., Gratton, M.A., Cosgrove, D. (2020) RNA-seq analysis of gene expression profiles in isolated stria vascularis from wild type and Alport mice reveals key pathways underling Alport strial pathogenesis. PLosOne15(8):e0237907.

Gratton, M.A., Rao V.H., Meehan, D.T., Askew, C. and Cosgrove, D. (2005) Matrix metalloproteinase dysregulation in the stria vascularis of mice with Alport syndrome: implications for capillary basement membrane pathology. Am J Path 166:1465-1474. PMCID 1606400.

Gratton, M.A., Smyth, B.J., Schulte, B.A. and Vincent, D.A., Jr. (1995) Na,K-ATPase activity decreases in the cochlear lateral wall of quiet-aged gerbils. Hear Res. 83:43-50.

Gratton, M.A., Smyth, B.J., Lam, C.F., Schmiedt, R.A. and Boettcher, F.A. (1997) Decline of the endocochlear potential correlates with decreasing Na,K-ATPase activity in the lateral wall of aged Mongolian gerbils. Hear Res. 108:9-16.

Meehan, D.T., Delimont, D., Dufek, B., Zallocchi, M., Phillips, G., Gratton, M.A., and Cosgrove, D. (2016) Endothelin-1 Mediated Induction of Extracellular Matrix Genes in Strial Marginal Cells Underlies Strial Capillary Basement Membrane Thickening in Alport Mice. Hear Res. 341:100-108.

Zhou Y, Liu F. (2022) Coordination of the AMPK, Akt, mTOR, and p53 Pathways under Glucose Starvation. Int J Mol Sci. ;23(23):14945.

Acknowledgments & Disclosures

The studies were conducted in the laboratories of Michael Anne Gratton at Washington University in St. Louis and at BoysTown National Research Hospital and that of Dominic Cosgrove at BoysTown National Research Hospital and were funded by NIH-NIDCD.

The authors would like to thank Abcam for their technical assistance with the ELISAs. The efforts of Grady Phillips, Ruth Gill of Washington University at Saint Louis and Skip Kennedy at Boystown National Research Hospital for their assistance in data collection and poster construction.

Brendan J Smyth, PhD, MD of Sanofi has no conflicts nor disclosures.