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Proteomics Analysis of Perilymph and Cerebrospinal Fluid in Mouse

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Abstract

Objectives—Proteins in perilymph may alter the delivery profile of implantable intracochlear drug delivery systems through biofouling. Knowledge of protein composition will help anticipate interactions with delivered agents.

Study Design—Analysis of mouse perilymph.

Methods—Protein composition of perilymph and cerebrospinal fluid (CSF) was analyzed using a capillary liquid chromatography-mass spectrometry-based iTRAQ quantitative proteomics approach. We searched against a mouse subset of the Uniprot FASTA protein database. We sampled perilymph from the apex of the mouse cochlea to minimize CSF contamination.

Results—More than 50 explicit protein isoforms were identified with very high confidence. iTRAQ reporter ions allowed determination of relative molar amounts of proteins between perilymph and CSF. Protein in perilymph was almost three times more concentrated than in CSF. More than one-third of the proteins in perilymph comprised protease inhibitors, with serpins being the predominant group. Apolipoproteins constituted 16%. Fifteen percent of the proteins were enzymes. Albumin was the most abundant single protein (14%). Proteins with relatively high perilymph/CSF ratios included broad-spectrum protease inhibitors and apolipoproteins.

Discussion—Some proteins found in perilymph, such as albumin and HMW kininogen, have been implicated in biofouling through adsorption to device materials. The relatively large quantities of apolipoprotein and albumin may serve as a reservoir for acidic and lipophilic drugs. Alpha-2-glycoprotein can bind basic drugs.

Conclusions—Perilymph is similar in protein composition to CSF, though amounts are 2.8 times higher. Protease inhibitors comprise the largest category of proteins.

Keywords

Intracochlear; drug delivery; biofouling; inner ear; protein

INTRODUCTION

Drug delivery to the inner ear is a growing field with current uses in the treatment and protection of the inner ear and potential future applications as regenerative therapies for hearing loss emerge. A major design consideration for implantable drug delivery systems is their ability to function properly while interacting with intrinsic biologic factors. Most implantable delivery systems for the inner ear are in direct contact with perilymph (reviewed in Swan et al.¹). Proteins in perilymph can adsorb onto the surfaces of the implant, which may lead to biofouling and changes in delivery profiles.^{2,3} Knowledge of protein composition of perilymph will help anticipate protein interactions with delivered agents and will allow implants and drug delivery regimens to be tailored for optimal performance through the device lifetime.

Previous analyses of proteins in perilymph were performed using electrophoretic techniques and candidate approaches to identification.^{4–7} With the advent of mass-spectrometry-based proteomics, tools now exist to separate and identify in small sample quantities unprecedented numbers of proteins and to automate the identification of explicit protein isoforms. We adapted Salt et al.'s⁸ perilymph collection procedures to the mouse to identify the most abundant proteins present in mouse perilymph and cerebrospinal fluid (CSF). The mouse was chosen because its proteome is relatively complete and because of its increasing importance as a genetic model for human disease.

MATERIALS AND METHODS

Sample Collection

CBA/CAJ male mice (Jackson Laboratories, Bar Harbor, ME) 3 months of age were anesthetized with a combination of 100 mg/kg of ketamine and 10 mg/kg of xylazine administered intraperitoneally. Anesthetic boosters (one-third the original dose) were administered every 20 minutes throughout the surgery. Procedures were performed in a heated (31°C) chamber. The surface of the tympanic bulla was exposed after making an incision extending from the mandibular symphysis to the clavicle. The digastric muscle was cut using a bipolar cautery. A wide opening in the bulla allowed access to the cochlear apex. An indelible pen was used to make a small mark on the apex of the cochlea where sampling would occur. Altering a method introduced by Salt et al.,⁸ the inner surface of the auditory bulla and the cochlea were coated with liquid cyanoacrylate glue and allowed to dry for 10 to 15 minutes. A thin layer of fingernail polish was applied on all surfaces within the bulla area to minimize contamination from surrounding tissues. An opening in the apical turn was created with a new 175-nm-diameter carbide drill (Drill Bit City, Prospect Heights, IL). Samples were collected with a drawn glass capillary tube. After discarding the first tube with approximately 100 nl of perilymph, an additional 0.5 μ l was collected and stored at –80°C for the proteomics analysis.

CSF was collected from one additional animal as described by Vogelweid et al.⁹ Skin and muscles layering the occipital bone and the atlas were removed, and the atlanto-occipital membrane was exposed, cleaned, and dried. To minimize contamination during CSF collection, a layer of fingernail polish was placed over the membrane and allowed to dry for 10 to 15 minutes. A 25-gauge needle was used to perforate the membrane and 1 μ l CSF was collected with a drawn glass capillary tube. The sample was stored at –80°C for analysis.

All procedures were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary.

Sample Preparation

Four samples were utilized for analysis: three perilymph samples of 0.5 μ l, each collected from a different mouse, and CSF from an additional mouse. The four samples were reduced,

alkylated, trypsin-digested, and derivatized with the 114, 115, 116, and 117 iTRAQ reagents¹⁰ using reagent solutions and following the standard protocol supplied with the iTRAQ™ kit (Applied Biosystems, Foster City, CA, Chemistry Reference Guide). Once the samples were separately labeled with iTRAQ reagents, they were combined and cleaned by strong cation exchange (SCX) using a 4×15 mm POROSTM 50-HS SCX cartridge (Applied Biosystems). Samples were applied to the SCX cartridge in 2 ml of load buffer (Applied Biosystems, 10 mM potassium phosphate, 25% acetonitrile [ACN], pH 3.0). The column was washed with 1 ml of load buffer, and the pass through was collected. The sample was then eluted using 500 μ l elution buffer (Applied Biosystems, 10 mM potassium phosphate, 375 mM potassium chloride, 25% ACN, pH 3.0). The SCX elution solvent was removed by vacuum centrifugation. The peptides were resuspended in 0.1% trifluoroacetic acid and cleaned on a C18 MicroSpin™ column (The Nest Group, South-borough, MA, Vydac C18 Silica). The eluant was dried in a speedvac.

Capillary Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LCMS/MS) Analysis

Data-dependent UPLC–MS/MS analyses were performed using a Waters nanoACQUITY UPLC® chromatography system and a Waters Q-TOF Premier™ MS system (Waters, Milford, MA). The combined sample (containing the three iTRAQ labeled perilymph samples and the CSF sample) was dissolved in 20 μ l of 2% ACN, 0.1% formic acid (FA) (solvent A), and quadruple analyses of the sample were performed (two with 5 μ l of each and two with 2.5 μ l of each) using the nanoACQUITY auto sampler. The peptides were first trapped on a 180 μ m ID × 20-mm trapping column packed with 5 μ m Symmetry® C18 (Waters) with 3% ACN, 0.1% FA as the mobile phase, at a flow rate of 5 μ l/min for 3 minutes. The flow was then reduced to 350 nl/min and directed through the analytical 75 μ m (ID) × 10 cm C18 capillary UPLC® column (130 Å, 1.7 μ m BEH130 nanoACQUITY®, Waters). A solvent gradient was run from 3% to 40% ACN in 0.1% FA in 110 minutes at a rate of 350 nl/min. Nanoelectrospray ionization was performed at 3,000 V with the heated capillary at 180°C. During the gradient elution, data-dependent scans were performed with four scan events per cycle consisting of one full MS from mass/charge ratio (m/z) 300 to 1,800 followed by product ion scans on the three most intense ions in the full scan using charge state optimized collision energy programs. Precursor ions used to obtain product ion scans were dynamically excluded (m/z –0.1, +0.1) from reanalysis for 20 seconds. Lock-mass spectra (glucosylated peptide, Sigma, St. Louis, MO) were acquired at 30-second intervals throughout the analyses.

Database Searching and iTRAQ Quantification

A Uniprot FASTA database containing all mouse protein sequences was downloaded from <http://www.informatics.jax.org/>, the mouse genome informatics website on July 31, 2008, and searched with Protein Lynx Global Server (PLGS) software (v2.2.5, Waters). Variable modifications that were searched for include: iTRAQ derivatives on N-term and lysine, oxidation of methionine, and alkylation of cysteine. The raw data were lock-mass corrected, de-isotoped, and charge state reduced by PLGS. Results were combined from four repeat LCMS analyses of the sample for data analysis. Determination of the iTRAQ reporter ion ratios was performed using the Expression Analysis routine in PLGS.

All proteins identified by the PLGS search engine were then individually evaluated by manual inspection. A concatenated target-decoy database analysis was also conducted to quantify the false positive rate.¹¹ Only one reversed sequence was found in the group of proteins identified by two or more peptides, resulting in an overall false positive rate of 1.75% for the reported protein identifications.

Protein Quantification

For each protein, relative content in the samples was based on determination of the exponentially modified protein abundance index (emPAI),¹² which estimates relative protein concentration based on the ratio of observed to observable unique peptides. The number of observable peptides per protein was determined by in-silico trypsin digestion of the identified proteins and counting of the resulting peptides that conformed to the range of HPLC retention values (calculated by the procedure of Brown et al.¹³) and molecular weight range observed from the list of all 443 peptides identified in all four analyses performed on the present set of samples. The iTRAQ reporter ion ratios provided relative ratios of each protein in the four samples analyzed. Assuming the protein content analyzed is the sum of the protein in each of the four contributing samples, we used an algebraic solution to determine the protein content in each of the four iTRAQ labeled samples. To quantify the relative amounts of each protein, we created a protein content index (P) for each of the four samples, where,

$$P_{\text{total}} = P_{\text{csf}} + P_{\text{peri1}} + P_{\text{peri2}} + P_{\text{peri3}}$$

iTRAQ reporter ion ratios provide the ratios of protein content in CSF to that in each of the perilymph samples, so

$$P_{\text{csf}} = P_{\text{total}} / (P_{\text{peri1}}/P_{\text{csf}} + P_{\text{peri2}}/P_{\text{csf}} + P_{\text{peri3}}/P_{\text{csf}} + 1)$$

Relative content of each protein in a sample is expressed on a molar basis as the mol%.

We did not centrifuge the samples after collection and consequently detected some hemoglobin in the proteomics analysis; still, none of the samples appeared to have a reddish hue. We excluded the hemoglobin content from our analysis.

RESULTS

Approximately 130 proteins were identified in the iTRAQ analysis of the sample that combined three perilymph and one CSF samples. From this data set, 50 proteins (Table I, with additional detail in the supplementary table) were identified by at least two peptides and were judged to have a >98% probability of correct identification by counting the number of segregate (reversed) proteins identified from the complete set of reversed sequences included in the database searched. The ratio of iTRAQ reporter ions allowed determination of relative amounts of each protein in each perilymph and CSF sample. The mass spectrometry-based proteomics methods used here provide a quantitative estimate of the amounts of each protein in the sample based on the ratio of observed to observable tryptic peptides identified for each protein.¹² Using both the estimated total amounts of each protein and the iTRAQ ratios, we were able to determine the relative amounts of each protein between each perilymph and CSF sample. The major proteins in perilymph and CSF were similar, though protein in perilymph was on average 2.8 times more concentrated than in CSF.

Albumin, the single protein found in highest concentrations in perilymph, accounted for around 14% of the protein in perilymph. Other proteins in relatively high concentrations (5%–6%) included prostaglandin D synthase (a lipophilic ligand binding protein¹⁴ thought to play a role in blood-organ barriers), two serine protease inhibitors (serpin a1d and serpin a1a), and two apolipoproteins (apoA2 and apoD). Figure 1 summarizes the major categories of proteins found in perilymph. The major family of proteins in perilymph is the group of protease inhibitors,

which represent more than one-third of the total protein content in perilymph. The predominant type of protease inhibitors were the serine protease inhibitors or serpins (27.8%), followed by several cysteine protease inhibitors (HMW kininogen, cystatin, and GUGU beta, 5.6%). The broad spectrum protease inhibitors (alpha-2-macroglobulin and murinoglobulin) comprised the remaining (3.1%) protease inhibitors. Protease inhibitors bind to and inactivate proteases. Although their specific role in the perilymph is unknown, a general role for these proteins is in mediating inflammation.

Enzymatic proteins represent another large family of proteins in perilymph, comprising more than 14% of the total. These include prostaglandin D synthase, carboxylesterase, carbonic anhydrase, lactate dehydrogenase, and several redox enzymes. Another major category of proteins in perilymph are the apolipoproteins, which act as lipid binders and transporters.¹⁵ Apolipoprotein D, A1, and A2 were relatively concentrated in perilymph compared with CSF. Apolipoprotein D may have a role in maintenance and repair in the central and peripheral nervous systems.

We also identified a number of other proteins of potential interest to auditory biologists, which did not meet our criteria for inclusion based on an automated probability analysis but were considered to be good identifications based on examination of individual spectra. These included cochlin, a secreted protein known to be involved in autosomal dominant nonsyndromal sensori-neural hearing loss (DFNA9);¹⁶ NF kappa B inhibitor; matrillin4, a protein involved in bone resorption; and malate dehydrogenase.

DISCUSSION

Protein Composition of Perilymph

Our results confirm and extend the analysis of Thalmann et al.,⁵ which was based on a candidate approach with 2D electrophoresis and which until now has been the most extensive analysis of perilymph proteins. We found that while protein concentration in mouse perilymph is higher than that in CSF, the composition is similar. Our data suggest protein concentration is 2.8 times higher in perilymph than in CSF, but this value should be taken only as an estimate, given that it is based on derived measures; the number of unique peptide fragments associated with each protein can be related to the quantity of protein injected but is not a direct measure of quantity injected. Our estimated perilymph/CSF ratio of 2.8 is low compared with analyses performed in other species. The ratio of total protein content in human perilymph to CSF has been reported to range from 4.3 to 5.^{5,6} Moreover, Thalmann found a perilymph/CSF protein ratio of 5 in human and 12.5 in guinea pig.^{4,5} The total protein in perilymph and CSF in guinea pig⁴ was 2.757 ± 0.238 and 0.219 ± 0.089 mg/mL $\pm$ SD, respectively. Thalmann's electrophoretic analysis of human and guinea pig perilymph identified several of the major proteins found in our analysis including albumin, alpha-1 antitrypsins (serpina1), antichymotrypsin (serpina3k), and serotransferrin.^{4,5} These were also identified in Arrer et al.'s⁷ study of human perilymph.

It is unlikely that we have significant contamination of our perilymph samples with CSF. We modified Salt's⁸ technique of collecting from the apical turn of the cochlea and collected only 0.5 μ l of fluid. Flow out of the apical cochleostomy was not as robust in the mouse as in the guinea pig, suggesting that the cochlear aqueduct may be less patent in the mouse than in the guinea pig. We suspect minor contamination with red blood cells in both the CSF and perilymph samples because we did detect hemoglobin in both, but we did not detect a number of other major proteins known to be present in mouse plasma, including apolipoprotein B, fibronectin, complement C5, and complement C4.¹⁷ We did not spin the sample before analysis, although we did not observe any red color in the fluid or in the dried protein. The extraordinarily high content of hemoglobin in red blood cells implies that even small amounts of contamination will be detected.

For any given protein, our estimates of differences between CSF and perilymph were based on the incorporation of iTRAQ reporter ions and are likely to be relatively accurate. We found murinoglobulin, alpha-2 macroglobulin, and some apolipoproteins (apoD, apoA1, and apoA2) to be major proteins that were 5- to 6-fold higher in perilymph than in CSF. The results confirmed Thalmann et al.'s earlier work in the guinea pig,⁴ which found high relative levels of apolipoprotein D compared with plasma and in human perilymph¹⁰ compared with plasma and CSF. Alpha-2 macroglobulin and murinoglobulin are both members of the I-39 family of protease inhibitors and inhibit all four families of proteases. Apolipoproteins are transporter proteins that can carry a variety of ligands. A couple of transcription-related proteins (BTB [POZ] domain protein and TAF7-like RNA polymerase) and liver carboxylesterase were also found to be elevated around five-fold in perilymph compared with CSF.

Implications for Inner Ear Drug Delivery

A concern with any implanted drug delivery device is the possibility of biofouling that can change the parameters of delivery over time. Protein adsorption to materials mediates the response of cells to the device as well. Biofouling limits the usefulness of implanted bio-sensors and can influence the inflammatory process.^{2,18} Extensive research on protein response to various materials is ongoing.^{19–21} Many studies use albumin, along with fibrinogen, as model proteins for understanding biofouling parameters.^{19–21} Because perilymph has a large amount of albumin, designs for implantable devices should incorporate biofouling considerations.

One known source for biofouling is high-molecular-weight kininogen, which is present in perilymph and is a factor in coagulation and inflammation.²² High-molecular-weight kininogen, produced in the liver, adsorbs to the surface of biomaterials that come in contact with blood *in vivo*.²³

Proteins found in perilymph may affect the metabolism and distribution of drugs delivered to the cochlea. Many proteins may bind to the drug molecules, making them unavailable to the target tissues. Albumin, alpha-1 acid glycoprotein, prostaglandin D synthase, and lipoproteins may act in this way. Albumin tends to bind acidic ligands. Alpha-1 acid glycoprotein, found in relatively small quantities in perilymph, is the primary carrier for basic drugs. Apolipoproteins and prostaglandin D synthase bind ligands with hydrophobic characteristics.¹⁵

Enzymes account for almost 15% of the protein in perilymph. Prostaglandin D synthase, found in relatively high concentrations in perilymph, is an enzyme that has, in addition to a variety of CNS functions, a role in maintaining the integrity of the blood-brain, blood-eye, and blood-testis barriers. A potential role in the blood-cochlear barrier is thus likely.

CONCLUSIONS

The protein composition of mouse perilymph is very similar to CSF, though proteins are on average 2.8 times more concentrated in perilymph than in CSF. Protease inhibitors comprise more than one-third of the total protein content in perilymph, and more than half of these are serine protease inhibitors. Albumin is the single protein present in highest amounts.

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Fig. 1.

Major protein groups identified in perilymph. Each group is represented as the percentage of the total amount of protein in perilymph. Albumin and alpha-2-glycoprotein are considered major ligand binding proteins for acidic and basic drugs, respectively. Apolipoproteins and prostaglandin synthase are capable of binding lipophilic drugs.

TABLE I
Major Proteins Identified in Mouse Perilymph Listed in Descending Order of the Molar Percentage in Perilymph.

Accession	Protein	Family or Function	Molecular Weight	Perilymph (mol%)	CSF (mol%)
ALBU_MOUSE	Albumin	Ligand binding	68647	11.9%	9.9%
PTGDS_MOUSE	Prostaglandin D synthase	Enzyme	21052	6.3%	6.7%
A1AT4_MOUSE	Serpin a1d	Serpin	45969	5.5%	4.8%
APOA2_MOUSE	ApoA2	Apolipoprotein	11311	5.3%	3.5%
APOD_MOUSE	ApoD	Apolipoprotein	21515	4.8%	2.7%
Q3KQQ4_MOUSE	Serpin a1a	Serpin	45867	4.3%	3.8%
A1AT2_MOUSE	Serpin a1b	Serpin	45945	4.2%	3.7%
A1AT1_MOUSE	Serpin a1a	Serpin	45973	4.1%	3.7%
A1AT5_MOUSE	Serpin a1e	Serpin	45862	3.8%	3.4%
A2APX3_MOUSE	Cystatin	Cysteine protease inhibitor	11133	3.1%	6.9%
A1AT6_MOUSE	Serpin a1f	Serpin	45794	3.2%	2.8%
TRFE_MOUSE	Serotransferrin	Iron binding	76673	3.2%	2.8%
Q58EV2_MOUSE	ApoA1	Apolipoprotein	23007	3.0%	1.8%
PRDX2_MOUSE	Peroxiredoxin	Enzyme	21765	2.6%	4.0%
Q6GTX3_MOUSE	ApoE	Apolipoprotein	35830	2.5%	3.7%
CTF2_MOUSE	Cardiotrophin 2	Cell signaling	21986	2.5%	2.8%
SPA3K_MOUSE	Serpin a3k	Serpin	46850	2.1%	2.0%
ACTB_MOUSE	Beta actin	Actin	41709	2.0%	3.0%
Q6S9I1_MOUSE	Hmw kininogen	Cysteine protease inhibitor	53171	2.0%	1.8%
VTDB_MOUSE	Vitamin D binding protein	Ligand binding	53565	1.5%	1.3%
A0AUP0_MOUSE	Ef hand C1	Calcium signaling	27995	1.5%	2.0%
A2M_MOUSE	Alpha 2 macroglobulin	I-39 protease inhibitor	165775	1.6%	0.8%
ESTN_MOUSE	Liver carboxylesterase	Enzyme	61133	1.5%	1.0%
MUG1_MOUSE	Murine globulin	I-39 protease inhibitor	165192	1.5%	0.7%
CO3_MOUSE	Complement C3		186364	1.3%	1.0%
Q9CS04_MOUSE	13 days embryo liver cDNA	Transcription coactivator	15919	1.3%	1.2%
HEMO_MOUSE	Hemopexin	Heme transporter	51308	1.2%	1.2%
PLMN_MOUSE	Plasminogen	Enzyme peptidase S1	90722	1.1%	1.0%
A2A513_MOUSE	Keratin 10	Keratin	57006	1.0%	2.2%

Accession	Protein	Family or Function	Molecular Weight	Perilymph (mol%)	CSF (mol%)
A2NHM3_MOUSE	If kappa		24149	1.1%	0.7%
Q8BWN0_MOUSE	Monoxygenase	Enzyme	13327	0.7%	1.5%
A1AG1_MOUSE	Alpha 1 acid glycoprotein	Ligand binding	23880	0.8%	0.8%
CAH2_MOUSE	Carbonic anhydrase	Enzyme	29014	0.7%	1.1%
B2RS10_MOUSE	GTPase activator	Enzyme activator	155179	0.7%	0.8%
FETUA_MOUSE	Fetuin	Cell signaling	37301	0.7%	0.7%
Q3TGJ9_MOUSE	Gelsolin	Actin binding	80798	0.5%	0.8%
Q6YJU1_MOUSE	GUGU beta	Cysteine protease inhibitor	33857	0.5%	0.5%
KCD13_MOUSE	BTB (POZ) domain protein	Transcription factor	36419	0.5%	0.2%
ANT3_MOUSE	Serpin c1	Serpin	51970	0.4%	0.5%
Q99K20_MOUSE	Lactate dehydrogenase	Enzyme	34481	0.4%	0.5%
CC113_MOUSE	Coiled-coil domain-containing protein 113		44187	0.4%	0.4%
B2ZP54_MOUSE	Phosphodiesterase	Enzyme	98834	0.3%	0.7%
K2C1_MOUSE	Keratin 1p	Keratin	65565	0.3%	1.0%
A2RTA0_MOUSE	Creatine kinase	Enzyme	43017	0.3%	0.6%
A1L0X5_MOUSE	Keratin 78	Keratin	54739	0.3%	0.8%
K2C73_MOUSE	Keratin 73	Keratin	58874	0.3%	0.9%
Q9CUG7_MOUSE	Adult male testis cDNA	Transcription coactivator	53017	0.3%	0.5%
ENOB_MOUSE	Beta enolase	Enzyme	46995	0.3%	0.4%
CERU_MOUSE	Ferroxidase	Enzyme	121074	0.3%	0.2%
Q3UV17_MOUSE	Keratin 2p	Keratin	62805	0.2%	0.5%

For each peptide, we have listed a family or function, although this categorization is arbitrary because most peptides can have multiple functions and families. CSF = cerebrospinal fluid.