Published in final edited form as:

Mol Cell Biochem. 2009 December ; 332(1-2): 215–224. doi:10.1007/s11010-009-0194-2.

Matrix imbalance by inducing expression of metalloproteinase and oxidative stress in cochlea of hyperhomocysteinemic mice

Soumi Kundu, Neetu Tyagi, Utpal Sen, and Suresh C. Tyagi

Department of Physiology & Biophysics, University of Louisville School of Medicine, 500 South Preston Street, Louisville, KY 40202, USA

Suresh C. Tyagi: suresh.tyagi@louisville.edu

Abstract

Clinical study reports hearing loss in patients with low folic acid (FA) and elevated homocysteine (Hcy). We hypothesize that elevated Hcy induces imbalance in matrix turnover and oxidative stress in cochlea. Cystathionine β -synthase heterozygous knockout mice were used as model for hyperhomocysteinemia. Matrix remodeling induced by Hcy resulted from elevated MMP-2, -9, and -14. MMP-2 and -9 showed elevated gelatinase activity in CBS ($\pm$) cochlea. Tissue inhibitors of matrix metalloproteinase were significantly lower in CBS ($\pm$) cochlea. The expression analyses for MMPs and TIMPs were equally represented at protein and mRNA levels. Cochlea of CBS mice showed following structural changes; (1) detachment of tectorial membrane lying on hair cells (2) thinner *s. vascularis* (3) large fibroblast in spiral ligament. Hcy induced higher protein nitrotyrosination and cytosolic NADPHoxidase subunit p22^{phox} in cochlea. It is thus suggested that Hcy induced matrix imbalance, structural changes and oxidative stress in cochlea.

Keywords

Cochlea; Homocysteine; Matrix metalloproteinase; Tissue inhibitors of metalloproteinase; Oxidative stress

Introduction

Clinical evidences suggest that age-related hearing loss is a common chronic condition in elderly. Certain percentage of hearing loss at higher frequencies has been attributed to circulatory disturbances as one of the main causes. Vascular compromise is a common occurrence associated with two independent risk factors, low serum folate levels, and elevated serum homocysteine (Hcy). Low folate status has been associated with poor hearing [1–3]. Folate is also one of the dietary determinants of serum homocysteine levels and lower folate levels cause ischemic vascular damage of the inner ear [4]. These clinical data suggest that elevated serum Hcy has a correlation with hearing impairment, the reason for which is still not well understood.

Hyperhomocysteinemia is implicated in a wide spectrum of disorders: vascular damage, cognitive impairment, and pregnancy complications [5,6]. A common underlying mechanism associated with vascular injury leads to these clinical manifestations. This includes oxidative damage of the endothelium through suppression of the vasodilator nitric oxide, promotion of platelet activation, and aggregation, alteration of the normal procoagulant–anticoagulant

balance promoting thrombosis, impaired methylation, and vascular smooth muscle cell proliferation [7,8]. Thus higher Hcy has a broad range of pathological conditions associated with it.

An elevated concentration of homocysteine associated with MTHFR gene point mutation as well as a polymorphism of the gene has a significant correlation with poor hearing [9]. Cohen-Salomon et al. (2007) [10] reported congenital hearing loss in *Cx30*^{-/-} (Connexin30 null) mice and disrupted vascular epithelium of the *stria vascularis* (SV). Their study also demonstrated that a localized high Hcy results in disrupted endothelial lining of the SV capillaries. Thus high plasma homocysteine may result in disruption of functional integrity of inner ear. Extracellular matrix (ECM) of inner ear is rich in collagen, which forms the basement membranes. DDR1 (Discoidin domain receptor 1) receptor, which is activated by collagen, when knocked out, leads to impaired functioning of inner ear [11]. Research in our laboratory has demonstrated that homocysteine regulates the composition and concentration of ECM [12–15].

Homocysteine is a thiol-containing amino acid produced during methionine metabolism and once formed it can be recycled to methionine by remethylation pathway or it can also be converted to cystathionine by cystathionine β -synthase (CBS), the first enzyme in the trans-sulfuration pathway [16,17]. Abnormal elevation of Hcy in plasma can be induced by several congenital and nutritional modifications that directly affects Hcy metabolism. The classical congenital disorder is that of cystathionine β -synthase deficiency, causing an elevation of plasma Hcy. A mouse model with heterozygous knockout of CBS (*CBS*^{+/-}) was developed by Watanabe et al. [18] which manifested mild hyperhomocysteinemia (HHcy). This model has been exploited by many investigators to study the pathophysiology of HHcy including endothelial dysfunction, oxidative stress, accelerated thrombosis [19–21].

The active matrix metalloproteinase (MMP) are enzymes, which degrade structural components of ECM. MMP proteolysis regulates tissue architecture through ECM and intercellular junctions, and they also affect intracellular functions via ECM mediated receptor activation [22,23]. MMPs are also regulated by their interaction with tissue inhibitors of matrix metalloproteases (TIMPs) [24,25]. The balance between MMPs/TIMPs thus maintains the integrity of the basement membrane and the conduction of signals for any physiological function. Homocysteine mediated remodeling also results in increased reactive oxygen species, reactive nitrogen species, and an overall increase in oxidative stress, which leads to endothelial dysfunction [26]. This study investigates the homocysteine related alterations in matrix composition in the cochlea. For this, we examined the change in expression level of MMPs and their regulators the TIMPs. We also investigated structural morphology by histological staining, and analyzed markers of oxidative stress in cochlea.

Materials and methods

Animals

Eight weeks old C57BL/6J and CBS ($\pm$) heterozygous knockout mice (created using same strain C57BL/6J) were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility center at University of Louisville. The mice were fed with regular mice chow PMI®LabDiet®St Louis, MO (Cat # 5015) and water ad libitum. Mice were genotyped for each group with a specific set of primers [16]. All animal procedures were performed in accordance with National Institute of Health Guidelines for animal research and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Antibodies and reagents

The following primary antibodies were used; anti MMP-2 (R&D systems, MN, Mineapolis), anti MMP-9 (Abcam Inc., Cambridge, MA); anti MMP-12 (H-300), anti MMP-13 (H-230), anti TIMP-1 (H-150), anti TIMP-3 (H-55), anti p22phox (FL-195), anti Nox3 (M-55), anti-Nitrotyrosine (7A5) were purchased from Santa Cruz biotechnology, CA, USA. Antibody to TIMP-2 was from Novus biological; monoclonal anti- β actin (Clone-74) from Sigma-Aldrich; antibody to MMP-14 was from Millipore. Secondary antibodies used were; rabbit pAb to goat IgG (HRP), goat pAb to rabbit IgG (HRP), goat pAb to mouse IgG (HRP) were from Abcam (Cambridge, MA). Histological staining kits used were Masson's trichrome stain-chromaview (Richard-Allan scientific, Kalamazoo, MI) and the histological mounting medium used was Permount[®] (SP15). PCR reagents used were as follows: 2X PCR master mix (PCR Master Mix, 2X: 50 U/ml of Taq DNA polymerase; 400 μ M dATP, 400 μ M dGTP, 400 μ M dCTP, 400 μ M dTTP, 3 mM MgCl₂ in reaction buffer).

Harvesting of mouse cochlear tissue

Cochleas from four animals were pooled for individual experiment and every experiment was repeated at least three times for the validation of results. The mice were deeply anaesthetized by overdose of injection of 2,2,2-Tribromo-ethanol (i.p). The anesthetized mice were transcardially perfused with cold PBS to flush out the blood and tympanic bulla was exposed by ventral approach. The tympanic bulla was clipped open and the bony capsule of the cochlea was removed under stereomicroscope. The cochlea was immediately washed in cold PBS and stored in -80°C for further use.

Isolation of protein

The bony cochlear capsule was dissected out and cochlear tissue from each group was collected in PBS on ice. For western blot analysis of matrix proteins, harvested tissue was pooled and minced finely in cacodylic acid buffer (10 mM cacodylic acid pH 5.0, 150 mM NaCl, 1 μ M ZnCl₂, 20 mM CaCl₂, 1.5 mM NaN₃, and 0.01% v/v Triton X-100). The minced tissue was kept on rotator at 4°C overnight for the extraction of proteins. The extract was centrifuged at $5,000\times g$ for 10 min and the supernatant was aliquoted and stored in -20°C for further use. For the isolation of total cellular proteins, the harvested cochlea was snap frozen in liquid nitrogen and grounded in $1\times$ RIPA buffer (Tris-HCl 50 mM, pH 7.4; NP-40, 1%; 0.25% Na-deoxycholate, 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μ g/ml each of aprotinin, leupeptin, pepstatin; 1 mM Na₃VO₄; 1 mM NaF) containing 1 mM PMSF and 1 μ g complete protease inhibitor (Sigma). The homogenate was kept on ice for 30 min, centrifuged at $10,000\times g$ at 4°C for 15 min and the supernatant was collected. Estimation of protein was done by BCA assay method (Pierce, Rockford, IL).

Western blotting

Western blot analysis for matrix associated proteins and oxidative stress marker was performed as follows. Briefly, protein was extracted using $1\times$ RIPA buffer (Tris-HCl 50 mM, pH7.4; NP-40 1%; Na-deoxycholate 0.25%; NaCl 150 mM; EDTA 1 mM; PMSF 1 mM; Na₃VO₄ 1 mM; NaF 1 mM; protease inhibitor cocktail 1 μ g/ml). A total of 15 μ g of protein was fractionated by SDS-PAGE and transferred onto PVDF membrane (BioRad, Hercules, CA) by wet transfer method. The transferred proteins were processed for immunodetection of specific antigens. Briefly, non-specific sites were blocked with 5% non-fat dry milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween- 20, pH 7.4) for 1 h at room temperature. The blot was then incubated with appropriate primary antibody in blocking solution according to the supplier's specific instructions. The blots were washed with TBS-T (3 times, 10 min each) and incubated with appropriate HRP- conjugated secondary antibody for 1 h at room temperature. After washing, ECL Plus substrate (Amersham Biosciences, Pittsburgh, PA) was applied to

the blot for 1 min. The blot was developed using X-ray film (RPI Corp, Inc., Mount Prospect, IL) with a Kodak 2000A developer (Eastman Kodak, NY). The blots were stripped and re-probed with β -actin. The immunoreactive bands were scanned and densitometrically analyzed Un-Scan-It software (Silk Scientific, Orem, UT).

Gelatin-zymography

Cochlea was harvested and cleaned minutely of any extra tissue attachment and from blood by flushing with PBS and observing under microscope. For each zymogram four cochlea from two animals was pooled (such experiment was repeated 3 times with different sets of animals) and homogenized in buffer containing 50 mM Tris-HCl [pH 7.6], 5 mM CaCl_2 , 150 mM NaCl, 0.05% Brij35 (Sigma, St. Louis, MO), 0.02% NaN_3 . The supernatant was collected, and estimation of protein was done using BCA assay Kit (Pierce, Rockford, IL). Aliquots containing 800 μg total protein were added to 80 μl gelatin-conjugated sepharose beads (Gelatin Sepharose 4B; Amersham Pharmacia Biotech) for affinity precipitation, and incubated overnight at 4°C in a rotator. The beads were rinsed three times with 500 μl TBS, transferred to 50 μl TBS in 10% DMSO, and incubated 30 min at 4°C. The supernatant containing eluted protein was then collected after centrifugation (1 min at 500 $\times$ g). Each sample was mixed with an equal volume of 2X zymography loading buffer (BioRad, Richmond, CA), incubated for 10 min at room temperature (RT), then separated on a 10% gelatin zymogram gel (BioRad, Richmond, CA). Gels were soaked in 1 $\times$ zymogram renaturation buffer for 15 min at RT; this was repeated three times. The gel was incubated in 1 $\times$ zymogram developing buffer for 24 h at 37°C, stained with Coomassie blue (1%) in a 30% methanol and 10% acetic acid 1 h, destained in water and documented.

Real time PCR

Total RNA was isolated from cochlear tissues harvested from mice using TRIzol reagent (Gibco BRL, Gaithersburg MD) following the instructions provided by the manufacturer. RNA samples that gave A260/A280 ~ 1.8–2.0 in the Nanodrop ND- 1000 UV - Vis spectrophotometer were utilized for study. RNA (2 μg) was reverse transcribed (RT) with oligodT primers using ImProm-IITM Reverse Transcription System (Promega, Madison, WI) using Oligo (dT)₁₅ primers in a total reaction volume of 20 μl . The RT program was 25°C for 10 min, 42°C for 50 min, and then 70°C for 15 min. The resulting cDNAs were diluted and were subjected to real-time PCR using SYBR green assay on Mx3000p QPCR (Stratagene). The reaction was carried out in duplicates with a final reaction volume of 25 μl containing 2 $\times$ Brilliant[®] SYBR[®] Green QPCR Master Mix (Stratagene), 300 nM of primer concentration, and 2 μl of the diluted cDNA. A standard was prepared using a serial dilution of a reference sample and was included in each real-time run to correct for possible variations in product amplification. Normalization was performed with GAPDH as internal reference gene. Melting curve analyses were performed at the end of the PCR to verify the identity of the products.

Primer design

Sequences for genes to be validated were accessed from NCBI <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide> and inserted into Primer 3 http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/ for primer design based on a criteria that included T_m values between 58 and 60°C, a length of 20 mer, a product size of 100–125 bp, with an absence of long G–C stretches. The details of primers employed along with the annealing temperatures and product size are provided in Table 1. Expression levels of GAPDH, MMP-2, MMP-9, MMP-12, MMP-13, MMP-14, TIMP-1, TIMP-2, and TIMP-3 were measured. Expression values were normalized to GAPDH and expressed as $2^{-\Delta C_T}$, where ΔC_T is the difference between the C_T values of the target transcript and GAPDH from the same tissue.

Processing, embedding and sectioning of mice cochlea

Mice from both experimental groups were anesthetized with an overdose of sodium pentobarbital. The ribcage of the animals was cut open and 20 ml of ice cold PBS was trans-cardially perfused, followed by 20 ml perfusion of 4% paraformaldehyde in 1× PBS. The temporal bone was exposed by ventral approach and the apical portion of the bony cochlea was gently opened using Dumont forceps, to allow the fixative to perfuse through the tissue. The cochlea was washed (10 × 3 min in 0.1 M PBS) and decalcified in 10% EDTA in 0.1 M PBS for 3 days at 4°C on a rotator. Cryoprotection was done by gradual infiltration of 10–30% sucrose in PBS before embedding. The cochlea was placed in OCT compound (TFM™ tissue freezing medium, Triangle biomedical, Durham, NC) carefully while observing under a dissecting microscope, for cryosectioning. The cochlea was oriented to obtain mid-modiolar cross-sections of the organ of corti. Sections were obtained from four different animals of each group. Eight micrometer thick sections were cut by cryostat, mounted on SuperFrost®/Plus slides (Fisher Scientific, Pittsburgh, PA) and post fixed in 4% paraformaldehyde for 10 min.

Histological staining

Masson's trichrome staining for collagen (blue) was performed on 8 µm thick cryosections from both groups as per manufacturer's instructions for Masson's trichrome stain-chromaview kit (Richard-Allan scientific, Kalamazoo, MI). The slides were examined under microscope (Olympus 1X81, Japan) at both 100× and 200× magnifications.

Statistical analysis

Results reported are representative of at least three independent repetitions. Data were statistically evaluated by Student's t-test (independent samples/2-tailed). We considered $P < 0.05$ to be statistically significant. Data are presented as $\pm$ SEM.

Results

Increased protein levels of MMP-2, -9, and -14 in CBS ($\pm$) as compared to wild type mice cochlea

To analyze changes in the level of matrix proteins due to elevated serum homocysteine, the expression level of proteins in wild-type was considered 100%, and percentage change for only significant variations in CBS ($\pm$) was calculated (Fig. 1a and b). In CBS ($\pm$) mice cochlea there was a highly significant increase in the protein levels of MMP-2, MMP-9, and MMP-14. Specifically, the active MMP-2 level showed a robust increase by ~70% and active MMP-14 levels were increased by ~22% (the antibody used for MMP-2 detected active MMP-2 active form at mol. wt. 62 kDa, and antibody used for MMP-14 detected both pro- and active forms, the pro MMP-14 band has not been shown here). The active MMP-9 was also significantly elevated by ~22% in the CBS ($\pm$) groups as compared to their WT counterparts. The increase in levels of MMP-12 and MMP-13 was not significant among the groups.

Tissue inhibitors of MMPs; TIMP-1, TIMP-2, and TIMP-3 showed a decrease in their protein levels in the CBS ($\pm$) mice

In order to know the possible cause of the increased level of the MMP-2, -9, and -14, their regulators, tissue inhibitors of matrix metalloproteinase, the TIMPs were analyzed. Thus TIMP-1, TIMP-2, and TIMP-3 protein levels were determined. All the TIMPs showed a highly significant decrease in their protein level in the CBS ($\pm$) groups as compared to their wild type counterpart (Fig. 2a and b).

Real time PCR for mRNA expression of matrix metalloproteinase and tissue inhibitors of matrix metalloproteinase

In order to know if the changes in the protein level of MMPs and TIMPs were due to regulation at mRNA expression real time PCR was performed. The results revealed that there was robust increase in the transcript level of MMP-9 in the CBS ($\pm$) mice cochlea (Fig. 3). The mRNA level of MMP-2 was also significantly higher in CBS ($\pm$) group. The analysis of TIMP-1, -2, and -3 at mRNA level showed a significant decrease in their expression (Fig. 3), as was true for their protein level.

Gelatin zymography revealed higher MMP-2 and -9 in CBS ($\pm$) cochlea as compared to wild type

The significant increase in the protein level of the gelatinase, i.e., MMP-2 and MMP-9 reasoned us to examine the pro-teolytic activity of the two proteases. The gelatin zymo-graphs showed both pro-MMP-2 (72 kDa) and pro-MMP-9 at 110 kDa along with the secreted active forms of MMP-2 and -9, respectively at ~ 66 and 92 kDa. Our results revealed higher gelatinase activity in cochlear extract of CBS ($\pm$) mice than WT group, though the amount of active MMP-2 was higher than active MMP-9 (Fig. 4).

Histological differences among CBS ($\pm$) and WT cochlea

Masson's trichrome staining was performed on 8 μ M thick cryosection of mice cochlea. To visualize the structural details of cochlea, Masson's trichrome stain was preferred, this enabled to obtain better contrast between different cellular structures and the morphological differences could be interpreted. The inner ear structures had following morphological changes; the tectorial membrane in CBS ($\pm$) was found to be detached from on top of the hair cells (Fig. 5a and b), the *s. vascularis* of the CBS ($\pm$) mice was thinner and the lining of the marginal epithelium was damaged (Fig. 6c and d). Certain cells inside the double layered epithelial *s. vascularis* appeared hypertrophied in CBS^{+/-} as compared to WT, as revealed from more intense (pink) cellular staining of the enlarged cells (Fig. 6a and b). Moreover, the spiral ligament area in CBS ($\pm$) group was less as compared to WT (Fig. 6c and d, denoted by capital letter SV).

Elevated level of homocysteine results in oxidative stress in cochlear structures

Increase in level of nitro-tyrosination (NT) of proteins results from elevated NO⁻. We compared the general pattern of change in the protein-NT between the groups using anti-3-nitrotyrosine antibody (Fig. 7a and b). We found a robust increase in the protein-NT level in the high molecular weight proteins (~ 80–95 kDa). This reasoned us to determine if there is any increase in the expression of NADPH Oxidase-3 which is responsible for generation of ONO⁻ species inside cell. Though we did not detect any change in its expression among the groups, but its cytoplasmic subunit, p22phox that is required for activation of the enzyme, showed a significantly higher expression in the CBS ($\pm$) group. This indicated that the enhanced expression p22phox up regulate NOX-3 enzyme activity, though protein level did not change.

Discussion

Elevated Hcy induces remodeling by disturbing matrix homeostasis in cochlear tissue

The genetic model of CBS heterozygous knockout mouse represented adequate model for analysis of elevated Hcy and pathological condition manifesting vascular compromise and endothelial dysfunction. These mice were thus, predisposed to HHcy due to their impaired ability to convert homocysteine to cystathionine. These mice when fed a typical control diet have elevated of plasma tHcy levels (≥ 10 μ mol/l compared to < 5 μ mol/l in wild-type, that means more than double) [27], which is an adequate model to investigate the effects of

hyperhomocysteinemia. The genotype and phenotype of heterozygous CBS ($\pm$) mice determination were performed as mentioned earlier [12]. The mechanism of pathological HHcy and related disorders in humans are well elucidated by using genetically disrupted CBS mice model. Cystathionine beta-synthase-deficient mice (CBS^{-/-}) rarely survive past the weaning age due to severe hepatic dysfunction. Due to very high mortality and severe pathological conditions of the CBS^{-/-} mice it had not been used for our experiment. Plasma Hcy of the null mice is often ~56 times higher than the wild type control whereas that of heterozygous knockout is $\geq$ two times that of WT. Under physiological conditions and high methionine diet a concentration of 300 μ M of plasma Hcy is most unlikely. Thus we have used heterozygous knockout mice, which are mild hyperhomocystenemic and manifest all pathological conditions of vascular pathogenesis and endothelial dysfunctions. Such a condition renders them vulnerable [28].

Our results demonstrated an increase in the level of MMP-2, -9, and -14 in the cochlea of CBS ($\pm$) mice. MMP-2 and -9 (collagenase) up-regulation occurs in inflammatory responses [22, 23]. HHcy related neurovascular, neurodegenerative, and neuroinflammatory damages are well documented. The prominent change induced by Hcy for vascular remodeling is by inducing the activity of MMP-2 and MMP-9 [14,15], which is also been demonstrated in our present study. The heterozygous CBS mice demonstrated vascular remodeling of carotid artery supplying to brain and induced significant vascular leakage [14]. Cerebrovascular dysfunction is induced by oxidative stress, this redox stress activates the endothelium and upregulates MMPs, which degrades the matrix and leads to blood–endothelial barrier leakage [29,30]. Homocysteine is known to induce inflammation in central nervous system by enhancing leakage through the blood–brain barrier (BBB) and compromising the cerebral microcirculation. A similar mechanism may also occur in cochlear microcirculation due to HHcy.

The structure of cochlea is composed of 12 different types of epithelial cells, which are involved in specialized functions. Basement epithelium supports the organ of corti on which rests the sensory epithelium and the vascular structure is composed of double layer epithelium involving four different types of epithelial cells. The intercellular matrices are rich in collagen and a homeostasis is maintained by regulating the turnover of matrix composition. Kidney related pathology of Alport's syndrome, which also shows manifestation of hearing inefficiency due to disturbance in homeostasis of matrix turnover and resulted in deposition of intercellular matrix in *s. vascularis* basement membrane [31]. MMP expression thus plays a crucial role reflecting cellular compensatory mechanism aimed at limiting the rate of matrix accumulation.

Cochlea has a rich vascular supply, which is embedded in a bed of double layer of epithelium, where the arterial supply breaks into minute capillaries. The endothelial cells lining the capillaries showed disruption resulting in leakage and such malfunctions have earlier been related to result due to elevated Hcy in SV [10]. Also, elastin is the most important cellular component of endothelium lining of blood vessels and capillaries. The metalloproteinase, MMP-12 is involved in tissue remodeling by degrading extracellular elastin, and has role in inflammatory responses and fibrosis. Even though we did not find any significant increase in MMP-12 in cochlea of HHcy mice, tissue remodeling at the vascular bed and endothelial disintegrity may not be ruled out based on elevated activities on other MMPs.

Since inner ear has a supporting structure of fibroblast cells, which surrounds the capillary beds, the expression analysis of MMP-13 was also taken into consideration due to its role in cartilage (chondrocyte, made up of fibroblast) remodeling. MMP-14 is the membrane type MMP and it activates MMP-2. Thus an increase in MMP-2 activity may result due to enhanced MT-1 MMP-14. The activation of MMP-2 (cleavage of the pro-form) involves the formation of a trimer composed of TIMP-2 binding to MMP-14 prior to association with pro-MMP-2.

So pro-MMP-2 activation occurs only at low TIMP-2 concentrations relative to MMP-14, which permits availability of active MMP-14 to activate the pro-MMP-2 bound in the ternary complex. The expression of TIMPs is also controlled during tissue remodeling and physiological conditions to maintain a balance in the metabolism of the extracellular matrix. Disruption of this balance may result in diseases associated with uncontrolled turnover of matrix.

Although different TIMPs bind tightly to most MMPs, some common inhibitory properties between different TIMPs are there. The association of proMMP-9 and TIMP-1 in a complex leads to the secretion of active MMP-9. Thus it was expected that the protein levels of the regulatory TIMPs will also determine the active MMP levels. Our results demonstrated a significantly reduced expression of TIMP-1, TIMP-2, and TIMP-3, which have overlapping inhibitory effects on the various MMPs. Although, the mechanism of homocysteine (Hcy) related cochlear dysfunction is not yet known, and Hcy may not have a direct effect on MMP and TIMP level. However, the change in MMP/TIMP ratio found in cochlea of CBS $\pm$ mice likely indicates cochlea damage. Interestingly, Cohen-Solomon et al. (2007) had demonstrated that hearing impairment connexin30 null mice have damaged *s. vascularis* with higher Hcy in cochlear circulation. So, it is likely that Hcy may have a crucial role in cochlear microcirculation and functioning. But the mechanism of Hcy induced cochlear function is not yet elucidated.

Gelatin zymograms revealed that in WT cochlea there is a constitutive basal level of gelatinase activity as is seen by the pro-MMP-2 and pro-MMP-9 levels (Fig. 4). Moreover active MMP-2 and MMP-9 were also detected in wild type cochlea. This may be explained as cochlea is rich in extracellular matrix and a regulated turn-over of the matrix is required for maintaining the cellular integrity and functioning of the organ. Densitometric determination of the active MMP-2 and MMP-9 revealed that there is a significant increase in the gelatinase activity of both the proteases.

Heterozygous CBS mice generated more superoxides in aortic tissue compared to controls [19]. Similarly, endothelial cell culture incubated with Hcy produce elevated ROS [3,32–34]. ROS consists of superoxide anions, hydroxyl radical, peroxynitrite, hydrogen peroxide, and other peroxides. ROS and oxidative stress promote the formation of nitrotyrosine, an indicator of NO and super-oxide radical reaction, resulting in the formation of the strong oxidant peroxynitrite. Peroxynitrite, besides other effects, leads to tyrosine nitration. This may alter protein function and induce cellular dysfunction. Our results demonstrated increased protein nitrotyrosination in CBS ($\pm$) cochlea which indicates possible cellular dysfunction. The protein nitrotyrosination also causes damage to electron-transport chain in the mitochondria and results in endothelial dysfunction. The distribution of nitric oxide in hair cells of cochlea and modulation of mitochondrial function has been demonstrated [35] in noise induced hearing loss. Such physiological changes in HHcy mice inner ear may be explained in the present study. We failed to notice any significant difference in the protein level of NADPH oxidase-3 (NOX3), but a variation in its enzymatic activity is expected as its subunit p22^{phox} showed a highly significant increase. NOX3 is a p22phox dependent enzyme, required for its activation [36].

Our results also demonstrated structural difference in cochlea among the groups. To visualize the cellular structure we specifically used masson's trichrome staining, which enabled the analysis of basement structure (collagen) in blue and cellular structures (red staining). The fibroblast population (type not determined) seen in Fig. 6b is hypertrophied (bigger in size) in HHcy group. The *s. vascularis* is also thinner in CBS ($\pm$) as compared to WT. The tectorial membrane in CBS group was detached from the hair cells whereas in WT the tectorial membrane was lying on top of the hair cells. It may be possible that due to limitation of

magnification used for microscopy, the other difference in structural details was not noted. But a compromise in other cellular structures between the groups may not be ruled out.

Thus, the elevated plasma Hcy induces changes in the cochlear structure not only by altering the matrix homeostasis, but also by increasing nitrosative stress.

Limitations—Inner ear expresses collagen I–V, IX, and the type II collagen is the most abundant. The staining procedure used for Collagen is Masson's trichrome which is not specific for any particular type of Collagen. Collagenase activity of MMP-9 and -2 is known to be associated with Col IV degradation. However, the inner ear has abundant collagen, and the turnover rate may be higher, and tightly regulated to ensure proper functioning. Moreover, imbalance in MMP/TIMP ratio is likely to cause change in collagen turnover and concentration; however it is likely that enhanced collagen degradation will be compensated by enhanced collagen synthesis. Thus the staining may not be a true representation of collagen abundance and decrease. Immunohistological staining using Col IV antibody will give quantitative picture of Col IV abundance in WT and CBS ($\pm$) groups.

Acknowledgments

A part of this study was supported by NIH grants-HL-71010; HL-74185; HL-88012; and NS-51568.

Abbreviations

CBS	Cystathione-beta synthase
MMP	Matrix metalloproteinase
TIMP	Tissue inhibitors of matrix metalloproteinase
Hcy	Homocysteine

References

1. Berner B, Odum L, Parving A. Age-related hearing impairment and B-vitamin status. *Acta Otolaryngol* 2000;120:633–637. [PubMed: 11039875]
2. Cadoni G, Agostino S, Scipione S, et al. Low serum folate levels: a risk factor for sudden sensorineural hearing loss? *Acta Otolaryngol* 2004;124(5):608–611. [PubMed: 15267180]
3. Durga J, Verhoef P, Anteunis LJC, et al. Effects of folic acid supplementation on hearing in older adults: a randomized, controlled trial. *Ann Intern Med* 2007;146(1):1–8. [PubMed: 17200216]
4. Fattori B, Nacci A, Casani A, et al. Hemostatic alterations in patients with acute, unilateral vestibular paresis. *Otolaryngol Head Neck Surg* 2001;124:401–407. [PubMed: 11283497]
5. Graham IM, Daly LE, Refsum HM, et al. Plasma homocysteine as a risk factor for vascular disease: the European concerted action project. *JAMA* 1997;277:1775–1781. [PubMed: 9178790]
6. Bautista LE, Arenas IA, Penuela A, Martinez LX. Total plasma homocysteine level and risk of cardiovascular disease: a meta-analysis of prospective cohort studies. *J Clin Epidemiol* 2002;55:882–887. [PubMed: 12393075]
7. Weiss N, Heydrick SJ, Postea O, Keller C, Keaney JF Jr, Loscalz J. Influence of hyperhomocysteinemia on the cellular redox state: impact on homocysteine-induced endothelial dysfunction. *Clin Chem Lab Med* 2003;41(11):1455–1461. [PubMed: 14656025]
8. Papatheodorou L, Weiss N. Vascular oxidant stress and inflammation in hyperhomocysteinemia. *Antioxid Redox Signal* 2007;9:1941–1958. [PubMed: 17822365]
9. Durga J, Anteunis LJC, Schouten EG, et al. Association of folate with hearing is dependent on the 5, 10-methylenetetrahydro folate reductase 677C-T mutation. *Neurobiol Aging* 2006;27(3):482–489. [PubMed: 16464657]

10. Cohen-Salmon M, Regnault B, Cayet N, et al. Connexin30 deficiency causes intrastrial fluid-blood barrier disruption within the cochlear stria vascularis. *Proc Natl Acad Sci USA* 2007;104(15):6229–6234. [PubMed: 17400755]
11. Meyer zum Gottesberge AM, Gross O, Becker-Lendzian U, Massing T, Vogel WF. Inner ear defects and hearing loss in mice lacking the collagen receptor DDR1. *Lab Invest* 2008;88(1):27–37. [PubMed: 18026164]
12. Kundu S, Kumar M, Sen U, et al. Nitrotyrosinylation, remodeling and endothelial-myocyte uncoupling in iNOS, cystathionine beta synthase (CBS) knockouts and iNOS/CBS double knockout mice. *J Cell Biochem* 2009;106(1):119–126. [PubMed: 19021146]
13. Sen U, Moshal KS, Singh M, et al. Homocysteine induced biochemical stress predisposes to cytoskeletal remodeling in stretched endothelial cells. *Mol Cell Biochem* 2007;302(1–2):133–143. [PubMed: 17525826]
14. Kumar M, Tyagi N, Moshal KS. Homocysteine decreases blood flow to the brain due to vascular resistance in carotid artery. *Neurochem Int* 2008;53(6–8):214–219. [PubMed: 18725259]
15. Kumar M, Tyagi N, Moshal KS, et al. GABAA receptor agonist mitigates homocysteine-induced cerebrovascular remodeling in knockout mice. *Brain Res* 2008;1221:147–153. [PubMed: 18547546]
16. Wang L, Jhee KH, Hua X, DiBello PM, Jacobsen DW, Kruger WD. Modulation of cystathionine beta-synthase level regulates total serum homocysteine in mice. *Circ Res* 2004;94:1318–1324. [PubMed: 15105297]
17. Jhee KH, Kruger WD. The role of cystathionine beta-synthase in homocysteine metabolism. *Antioxid Redox Signal* 2005;7:813–822. [PubMed: 15890029]
18. Watanabe M, Osada J, Aratani Y, et al. Mice deficient in cystathionine beta-synthase: animal models for mild and severe homocyst(e)inemia. *Proc Natl Acad Sci USA* 1995;92:1585–1589. [PubMed: 7878023]
19. Eberhardt RT, Forgione MA, Cap A, et al. Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest* 2000;106:483–491. [PubMed: 10953023]
20. Hofmann MA, Lalla E, Lu Y, et al. Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *J Clin Invest* 2001;107(6):675–683. [PubMed: 11254667]
21. Dayal S, Wilson KM, Leo L, et al. Enhanced susceptibility to arterial thrombosis in a murine model of hyperhomocysteinemia. *Blood* 2006;108(7):2237–2243. [PubMed: 16804115]
22. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodeling. *Nat Rev Mol Cell Biol* 2007;8(3):221–233. [PubMed: 17318226]
23. Moshal KS, Metreveli N, Frank I, Tyagi SC. Mitochondrial MMP activation, dysfunction and arrhythmogenesis in hyperhomocysteinemia. *Curr Vasc Pharmacol* 2008;6(2):84–92. [PubMed: 18393909]
24. Raffetto JD, Khalil RA. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Curr Vasc Pharmacol* 2008;6(3):158–172. [PubMed: 18673156]
25. Chase AJ, Newby AC. Regulation of matrix metalloproteinase (matrixin) genes in blood vessels: a multi-step recruitment model for pathological remodelling. *J Vasc Res* 2003;40(4):329–343. [PubMed: 12891002]
26. Weiss N, Heydrick S, Zhang YY, Bierl C, Cap A, Loscalzo J. Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathionine β -synthase-deficient mice. *Arterioscler Thromb Vasc Biol* 2002;22:34–41. [PubMed: 11788458]
27. Sen U, Basu P, Abe OA, et al. Hydrogen sulfide ameliorates hyperhomocysteinemia associated chronic renal failure. *Am J Physiol Renal Physiol*. 2009;10.1152/ajprenal.00145.2009
28. Jiang X, Yang F, Tan H, Liao D, Bryan RM Jr, Randhawa JK, Rumbaut RE, Durante W, Schafer AI, Yang X, Wang H. Hyperhomocysteinemia impairs endothelial function and eNOS activity via PKC activation. *Arterioscler Thromb Vasc Biol* 2005 Dec;25(12):2515–2521. [PubMed: 16210565]
29. Tyagi SC, Lominadze D, Roberts AM. Homocysteine in microvascular endothelial barrier permeability. *Cell Biochem Biophys* 2005;43(1):37–44. [PubMed: 16043881]
30. Lominadze D, Roberts AM, Tyagi N, et al. Homocysteine causes cerebrovascular leakage in mice. *Am J Physiol Heart Circ Physiol* 2006;290(3):H1206–H1213. [PubMed: 16258031]

31. Gratton MA, Rao VH, Meehan DT, et al. Matrix metalloproteinase dysregulation in the stria vascularis of mice with Alport syndrome: implications for capillary basement membrane pathology. *Am J Pathol* 2005;166:1465–1474. [PubMed: 15855646]
32. Zhang X, Li H, Jin H, et al. Effects of homocysteine on endothelial nitric oxide production. *Am J Physiol Renal Physiol* 2000;279:F671–F678. [PubMed: 10997917]
33. Tyagi N, Sedoris KC, Steed M, et al. Mechanisms of Homocysteine induced oxidative stress. *Am J Physiol Heart Circ Physiol* 2005;289(6):H2649–H2656. [PubMed: 16085680]
34. Postea O, Krotz F, Henger A, et al. Stereospecific and redox-sensitive increase in monocyte adhesion to endothelial cells by homocysteine. *Arterioscler Thromb Vasc Biol* 2006;26:508–513. [PubMed: 16373615]
35. Shi X, Han W, Yamamoto H, et al. Nitric oxide and mitochondrial status in noise-induced hearing loss. *Free Radic Res* 2007;41(2):1313–1325. [PubMed: 17963121]
36. Heydrick SJ, Weiss N, Thomas SR, et al. L-Homocysteine and L-homocystine stereospecifically induce endothelial nitric oxide synthase-dependent lipid peroxidation in endothelial cells. *Free Radic Biol Med* 2004;36:632–640. [PubMed: 14980706]

Fig. 1.

Expression profile of matrix metalloproteinases shows increased MMP-2, -9, and -14 in CBS (±) as compared to WT. Protein level of MMPs in wild type and CBS (±) mice cochlea was compared. **a** Representative western blot of MMP-2, MMP-9, MMP-12, MMP-13, MMP-14, and β-actin from groups was loaded in duplicate. Two lanes from each group represented one repetition (pooled tissue from four mice). Experiment was done thrice with different batches of animals. **b** Data are represented as histograms quantified as the integrated density values (IDV=pixel intensity) of each immunoreactive band normalized with β-actin (IDV of band of interest/IDV of β-actin). Data are expressed as the mean (±SEM) of the normalized values and

differences between means were determined by the Student *t*- test (* significant difference at $P < 0.05$)

Fig. 2.

Down-regulation of TIMP-1, -2, and -3 protein expression in CBS ($\pm$) cochlear tissue as compared to WT. Extraction of cochlear matrix associated protein was done and analyzed for TIMP-1, TIMP-2, and TIMP-3 protein levels which are associated with inhibitory activity of matrix proteases. **a** Representative western blots showing TIMP-1, -2, -3, and β -actin protein level in cochlear tissue of WT and CBS ($\pm$) mice. **b** Histogram of mean ($\pm$ SEM) of the IDV of band intensity for protein of interest/IDV of β -actin from three independent experiments. Difference among the groups was determined by Student *t*-test with significance level set at $P < 0.05$

Fig. 3.

Messenger RNA levels of MMP-2, -9 were up-regulated, whereas TIMP-1, -2, and -3 were reduced in CBS ($\pm$) as compared to WT. Data analyses for real time PCR were done by $\Delta\Delta CT$ method. Endogenous control for each assay was taken as the level of GAPDH mRNA. To express the fold change in expression for a particular gene, the WT levels were taken as 1 and $2^{-(\Delta C)}$ values for CBS was calculated and plotted as histograms. MMP-9 and MMP-2 mRNA levels are significantly up regulated, as reflected at their protein levels. Similarly TIMP-1, -2, and -3 are significantly down-regulated both at protein (Fig. 4) level

Fig. 4. Gelatinase activity of MMP-2 and MMP-9 is higher in the CBS ($\pm$) cochlea. The proactive form of MMP-9 and MMP-2 was detected at a basal level. The secreted zymogens are active MMP-2 and active MMP-9 forms, which are higher in CBS group than WT group. Each zymogram represented extract from four cochleas (two animals), which was repeated three times with different sets of animals

Fig. 5.

The organ of Corti showed structural differences between WT and CBS ($\pm$) cochlea. **a** tectorial membrane lying on top of the hair cells of the organ of Corti (*arrow*). **b** CBS ($\pm$) mice cochlea showed a detached tectorial membrane from on top of the hair cells. 100 $\times$ magnification

Fig. 6. Comparison of histological differences between WT and CBS ($\pm$) mice cochlea as revealed by Masson's trichrome staining: **a** & **b** Fibroblast areas (fibroblast type not determined) showed enlarged cells in CBS ($\pm$) group. **c** & **d** *s. vascularis* (SV) of hyperhomocysteinemic (HHcy) mice was thinner. Pictures shown are taken at 100 $\times$ and 200 $\times$ magnification

Fig. 7. Elevated Hcy causes induced protein Nitrotyrosination and p22^{phox} levels indicating higher oxidative stress. **a** Representative Western blot showing protein levels of NT, Nox-3, p22^{phox}, and beta actin in cochlear tissue **b** data represented as histograms of the normalized integrated density value (IDV in pixels) of immunoreactive bands

Table 1

Gene specific primer pairs used for real time PCR amplification

Gene	Accession No. (NCBI)	Primer pair	Length of product (bp)
GAPDH	NM_008084	5' CAACAGCAACTCCCACTCTT 3' 5' TGTTGCTGTAGCCGTATTCA 3'	110
MMP-2	NM_008610.2	5' GACACCTGCACCACCTTAAC 3' 5' GATGTCAGACAACCCGAGTC 3'	116
MMP-9	NM_013599	5' CATGTCACCTTTCCTTCACC 3' 5' TTGCCGTCCTTATCGTAGTC 3'	115
MMP-12	NM_008605	5' GTGCTTGCTGGTTTTTCAGT 3' 5' CACCTCCCCTTATTTCTGT 3'	104
MMP-13	NM_008607	5' CCATTTCATTGCTTCCTGATG 3' 5' GATAGGGCTGGGTCACACTT 3'	117
MMP-14	NM_008608	5' TTCTTTTCCATCCCCCTAAC 3' 5' GAGGCAGCCAAGGTACA 3'	108
TIMP-1	NM_001044384	5' CCAGAAATCAACGAGACCAC 3' 5' TTTCCATGACTGGGGTGTAG 3'	124
TIMP-2	NM_011594	5' AAGAATCACCTCCAACCAG 3' 5' CTTGGTTGAAGCCAGAAACA 3'	116
TIMP-3	NM_011595	5' GCCCTCCCATATGTATACCC 3' 5' TAGGCCTCACCTCAAGTCTG 3'	112