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Molecular mechanisms underlying cochlear degeneration in the tubby mouse and the therapeutic effect of sulforaphane

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ABSTRACT

As with Usher syndrome observed in humans, the two main phenotypes of the tubby mouse are progressive hearing loss and retinal degeneration. Yet, the mechanism underlying the tub-related cochlear degeneration is still unclear. The reduction/oxidation (redox) imbalance in the cell is related to many kinds of diseases. This study examined expressions of thioredoxin (Trx) and Trx reductase (TrxR), an important redox system in the cell, and the related upstream and downstream proteins of the Trx/TrxR in the tubby mouse cochlea. This report also examined the therapeutic effect of sulforaphane (SF) on the cochlear degeneration, which showed a protective effect on the tub-related retinal degeneration in our previous report. The results showed that the tub-mutation resulted in a significant suppression of Trx and TrxR expressions. Expression level of Nrf2 (NFE2 related factor 2), a transcription factor that regulates expression of Trx and TrxR and others, was also suppressed in the tubby mouse cochlea. Furthermore, a lowered level of activated extracellular signal-regulated kinase (p-ERK) was observed in the tubby mouse cochlea. In contrast, caspase-3 expression and activity were enhanced in the tubby mouse, suggesting apoptotic cell death. The tub-related molecular alterations in the cochlea were prevented by chronic treatment with SF. As a result, the SF-treatment significantly delayed the tub-related cochlear degeneration. Other unknown proteins may contribute to tubby-related degeneration because Nrf2 regulates many other antioxidants besides Trx/TrxR and sulforaphane did not prevent cochlear degeneration completely although it completely prevented alterations of Nrf2 and Trx/TrxR.

1. Introduction

The tubby strain of obese mice arose spontaneously in a mouse colony at the Jackson Laboratory (Coleman and Eicher, 1990). It is an autosomal recessive mutation, mapping to mouse chromosome 7 (North et al., 1997). The mutation has been associated with a G → T transversion, which abolishes a donor splice site in the gene and results in a larger transcript containing the unspliced intron (Noben-Trauth et al., 1996). The tubby phenotype is characterized by late-onset weight gain accompanied by progressive cochlear and retinal degeneration (Ohlemiller et al., 1995, 1997). The combination of these phenotypes resembles human syndromes, such as Usher's (retinal and cochlear degeneration), Bardet-Biedl, and Alstrom's (obesity and sensory deficits). The tubby is a loss-of-function mutation of the tub gene and that loss of the tub gene is sufficient to give rise to the full spectrum of tubby phenotypes (Stubdal et al., 2000).

A progressive hearing loss was reported in the tubby mouse after 3 weeks of age (Heckenlively et al., 1995; Ikeda et al., 1999). A slightly delayed cochlear degeneration was also reported (Ohlemiller et al., 1995, 1997). The mutation caused outer hair cell (OHC) loss in the extreme basal region (hook area) by 1 month of age and the damaged area expanded to the apical turn by about 6 months of age. The inner hair cells (IHCs) were affected in the hook region by

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Abbreviations: ARE, antioxidant-responsive element; ASK1, apoptosis signal-regulating kinase 1; CBB, Coomassic brilliant blue R-250; C_t, threshold cycle; ERK, extracellular signal-regulated kinase; IHC, inner hair cell; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NADPH, reduced form of nicotinamide-adenine dinucleotide phosphate; NFE2, nuclear factor E2; Nrf2, NFE2-related factor 2; OHC, outer hair cell; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; Redox, reduction/oxidation; ROS, reactive oxygen species; SDH, succinate dehydrogenese; SF, sulforaphane; TNBTZ, tetranitro blue tetrazolium; Trx, thioredoxin; TrxR, thioredoxin reductase; tub/tub, homozygous mutant tubby mice; tub/wt, heterozygous tubby mice; wt/wt, wild type C57BL/6J mice.

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about 6 months of age (Ohlemiller et al., 1995, 1997). Yet, the mechanism underlying the death of auditory hair cells in the tubby mouse is still unclear.

Reduction/oxidation (redox) imbalance is related to many kinds of diseases (Fujino et al., 2006). Thioredoxin (Trx) and Trx reductase (TrxR), with NADPH, compose an important redox system in the cell (Buchanan et al., 1994; Fujino et al., 2006; Holmgren, 1995). Trx is characterized by a redox active site with the sequence of -Trp-Cys-Gly-Pro-Cys-Lys-. The two cysteine residues within the redox active center provide the sulfhydryl groups involved in reducing activity. The Trx can be oxidized by free radicals, such as reactive oxygen species (ROS). The oxidized form Trx (Trx-S_2), containing a disulfide bridge in the active site, is reduced to a dithiol (Trx-$(SH)_2$) by TrxR in the presence of NADPH. Besides functioning as an antioxidant, Trx has also been identified as an interacting partner or a physiological inhibitor of ASK1 (apoptosis signal-regulating kinase 1), which is a mitogen-activated protein kinase kinase kinase (MAP3K), and activation of which initiates cellular stress response signaling cascades including JNK and p38 pathways (Ichijo et al., 1997; Roberts and Der, 2007). The reduced form of Trx binds to and inhibits ASK1 and the subsequent activation of JNK/p38 activities and apoptotic processes (Saitoh et al., 1998). In contrast, oxidization of Trx by ROS releases Trx from ASK1 leading to JNK and p38 activities and then caspase activation and apoptotic cell death.

Nuclear factor erythroid 2 (NFE2)-related factor-2 (Nrf2) is a transcription factor that regulates the expression of Trx and TrxR as well as other antioxidants via the antioxidant response element (ARE) in the nuclear DNA (Copple et al., 2008). In the absence of cellular stress, Nrf2 is tethered within the cytosol by an inhibitory partner, Keap1. ROS accumulation or activation of extracellular signal-regulated kinase (ERK) releases Nrf2 from the complex. Translocation of the Nrf2 from cytosol to the nucleus activates ARE-regulated gene expression (Copple et al., 2008).

Sulforaphane (SF), a naturally occurring isothiocyanate (Zhang et al., 1992), showed a protective effect on the tub-related retinal degeneration observed in our previous report (Kong et al., 2007). The SF-treatment was repeatedly reported to enhance Trx and TrxR expressions (Kong et al., 2007; Tanito et al., 2005; Zhang et al., 2003). This experiment was designed to determine expressions of Trx, TrxR, and the related upstream/downstream proteins in the tubby mouse. This experiment also explores the therapeutic effect of SF in preventing tub-related cochlear degeneration.

2. Materials and methods

2.1. Animals and genotype analysis

Homozygous mutant tubby mice (tub/tub) were purchased from the Jackson Laboratory (Bar Harbor, ME), and bred with wild type C57BL/6J (wt/wt) mice in the University of Oklahoma Health Sciences Center animal facilities. The obtained heterozygous tubby mice (tub/wt) were used as breeding parents. All animals were born and raised in a 12-h-on and 12-h-off cyclic light environment. The animal facilities are registered with the US Department of Agriculture and are inspected semiannually by the members of the Institutional Animal Care and Use Committee (IACUC) serving the University of Oklahoma Health Sciences Center. All procedures regarding the use and handling of animals were reviewed and approved by the IACUC. For genotype analysis, we used the Jackson Laboratory protocol (Kong et al., 2006, 2007). Briefly, genomic DNA was purified by proteinase K digestion and 2-propanol extraction from tail snips for PCR. Primer sequences used were 5′-CCGTGTCACA-CAGGCTTCT-3′ (forward); and 5′-CTGGGCACCATGCGTACA-3′ (reverse). PCR conditions consisted of denaturation at 94 °C for 1.5 min, then 35 cycles of 94 °C, and 55 °C, and 72 °C for 30 s, a final extension at 72 °C for 2 min, and 10 °C for 10 min. The PCR products were digested with the SmlI restriction enzyme (New England BioLabs, Inc., Beverly, MA) at 55 °C for 2 h and then separated by 5% MetaPhor agarose gel (FMC, BioProducts, Rockland, ME) electrophoresis. In the wt/wt mice, the 101 bp PCR products were undigested. In the tub/tub mice, the 101 bp PCR products were cut into 52 bp and 49 bp products. In the tub/wt mice, some PCR products were cut into 52 bp and 49 bp products and some were undigested (see Fig. 1). A total of 169 mice (including wt/wt, tub/wt/, and tub/tub) were used in this study. Animal number in each experiment in each group was present in the caption for each figure.

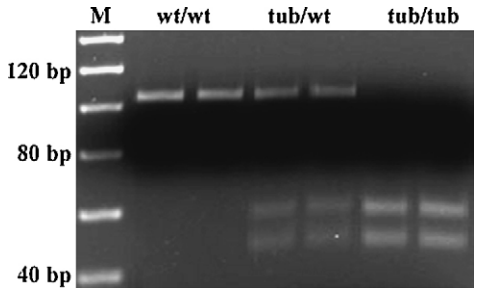

Fig. 1. Genotypes of the mice used in this study. Wild type (wt/wt): characterized with 101 bp PCR product; homozygous mutant type (tub/tub): characterized with 52 bp and 49 bp 2 digested PCR products; heterozygous type (tub/wt): characterized with all of the 3 undigested and digested products.

2.2. Sulforaphane (SF) treatment

Mice at postnatal day 10 (P10) were injected intraperitoneally (i.p.) with SF (S8046, LKT Laboratories Inc., St. Paul, MN) at a dose of 50 mg/kg body weight (dissolved in phosphate buffered saline (PBS)), or PBS alone for 20 days (P10–P30, 1/day) as controls. The SF-dose was chosen based on our previous study in which the SF-treatment prevented the tub-related molecular alterations and delayed degeneration in the retina (Kong et al., 2007).

2.3. Hair cell counting

Animals were anesthetized with CO_2 and then decapitated and the cochleae were removed immediately. The round and oval windows, as well as the apex of the cochlea were opened to facilitate perfusion. The cochleae were perfused with the incubation solution containing 0.05% tetranitro blue tetrazolium (TNBTZ), 0.05 M sodium succinate, and 0.05 M phosphate buffer and then incubated in the solution for 1 h at 37 °C. TNBTZ is an electron acceptor that, on reduction, precipitates as an insoluble and highly colored formazan. Succinate dehydrogenese (SDH) in the cell oxidizes sodium succinate and provides electrons for the reduction of the electron acceptor. Thus, the cell is colored. The stained cochleae were fixed in 10% buffered formalin for 2 days. After fixation, the cochleae were decalcified in 7% EDTA (ethylenediamine tetraacetic acid) solution. The basilar membranes with the organs of Corti were dissected out and mounted on slides. Hair cells within each section of 500-μm-length on the basilar membrane were counted under a light microscope and the cell numbers were compared to the control numbers obtained in the wild type mice and expressed as % of loss. Hair cell losses were plotted as a function of cochlear length (cochleogram).

2.3.1. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Kong et al., 2007; Zhou et al., 2005). Briefly, mice were decapitated after anesthesia with CO_2 inhalation and the cochleae were removed immediately. The cochleae were frozen in the liquid nitrogen and stored in a −80 °C freezer before RNA extraction. Total RNA from two cochleae of each mouse was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. The concentration of RNA was quantified by reading the optical density at 260 nm on spectrophotometer (Bio-Rad, Hercules, CA). cDNA was synthesized using The First-Strand Synthesis System for RT-PCR kit (Invitrogen Corp., Carlsbad, CA). PCR conditions consisted of a denaturation at 94 °C for 2 min, then 26–30 cycles of 94 °C for 30 s, 55–58 °C for 15 s, 72 °C for 30 s, and the final extension at 72 °C for 10 min. The PCR products and DNA marker were loaded on agarose gel (1–2% in 1× TAE buffer, Invitrogen Corp., Carlsbad, CA) and electrophoresed at 105 V for 0.5–1 h. The PCR products on agarose gel were visualized by staining with 0.5 μg/mL ethidium bromide and the gel was photographed under UV light. Gray scale of the band was measured, averaged within each group, and compared between groups. Internal control housekeeping gene, 18S, was run simultaneously. The primer sequences used for PCR were as follows (forward and reverse):

18S: (accession no. NR_003278):
5′-GTAGTGACGAAAAATAACAATACA-3′ and 5′-TGCTGGCACCAGACTTGCCCT-CCA-3′
Trx (accession no. X77585):
5′-CAAATGCATGCCGACCTTCCAGTT-3′ and 5′-TGGCAGTTGGGTATAGACTCT-CCA-3′
TrxR (accession no. NM_015762):
5′-TGTAATGGTGCGGTCCATTCTCCT-3′ and 5′–TTGTGGATTGAGCAGT-CACCCTGA-3′
Nrf2 (accession no. U20532):
5′-AGTTCTCGCTGCTCGGACTA-3′ and 5′-AGGCATCTTGTTTGGGAATG-3′
Caspase-3 (accession no. NM_009810):
5′-TGGCAACGGAATTCGACTCCTTCT-3′ and 5′-TGAGCATGGACACAATA-GACGGGA-3′