Hearing analysis in heterozygous and homozygous klotho gene deficient mice

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ARTICLE INFO

Article history:
Received 29 January 2018
Received in revised form 18 April 2018
Accepted 18 April 2018

Keywords:
Homoygous
klotho gene
Hearing loss

ABSTRACT

Objective: To understand the crucial role of the klotho gene in hearing development in mouse models.
Methods: PCR was used to identify CBA mice with different genotypes, i.e. WT, heterozygous (klotho +/−) or homozygous (klotho −/−). Mice phenotype and weight were recorded postnatal 25 days (P-25) and auditory brainstem responses (ABR) were used to determine auditory function at P-60.
Results: klotho −/− mice tended to have smaller size, lighter weight and higher ABR thresholds at P-60, showing early onset age-related hearing loss (ARHL).
Conclusion: Heterozygous and homozygous klotho deficient mice exhibit different degrees of hearing loss at young age, with homozygous mice (klotho −/−) showing more severe hearing loss. Our results indicate that persistent expression of klotho protein in the inner ear may potentially delay the onset of ARHL and play an important role in the protection of auditory function.

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1. Introduction

Age-related hearing loss (ARHL) or presbycusis is a gradual hearing degeneration (Tavanai and Mohammadkhani, 2016), during which hair cells in the cochlea and auditory nerves lose their capabilities to sense sound and conduct nerve signals. ARHL typically starts at high frequencies and later spreads to mid frequencies. Epidemiological studies show that ARHL first shows up among people aged from 40 to 60 years, and the incidence rises markedly as people grow older (Yamasoba et al., 2013). Nearly 50% of people aged over 70 suffer from this disorder (Fransen et al., 2003)(Huang and Tang, 2010). Latest data indicate that by 2030 the number of senior citizens suffering from ARHL may increase to 40–50 million in USA (Gates and Mills, 2005). These elderly patients will have to face all sorts of problems everyday as a result of natural deterioration of hearing that potentially can lead to compromised ability to recognize language in noisy environment (Ciorba et al., 2012). More worryingly, depression, anxiety and gradual loss of cognitive function may soon follow and will seriously affect the health of ARHL patients whose compromised hearing prevents them from interacting with the outside world (Dalton et al., 2003; Heine and Browning, 2002; Schuknecht, 1964).

Some recent research shows that the anti-aging Klotho protein may be closely associated with many senile diseases (Kuroo et al., 1998). For example, Kamemori et al. reported that Klotho expression was featured in cochlear stria vascularis and spiral ganglion neurons, and therefore inferred that the protein might have played a role in maintaining inner ear ion balance (Kamemori et al., 2002). Further research revealed that auditory brainstem response (ABR) thresholds in klotho ± mice were significantly higher than those in wild type (WT) mice with a latency delay in Wave I, an indication of hearing impairment (Takumida et al., 2009). The results suggest that the Klotho protein is involved in the formation of the inner ear and plays an important role in the protection of hearing function.

https://doi.org/10.1016/j.joto.2018.04.001
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Although heterozygous klotho deficient mice exhibit higher ABR thresholds evoked by clicks, frequency specific defects in these mice are yet to be established. Moreover, since homozygous (klotho \(-/-\)) mice are infertile and have shorter life expectancy (between P-50 to P-75), few studies have focused on testing the hearing ability in klotho \(-/-\) mice.

In this study, we are interested in frequency specific auditory defects and most vulnerable frequencies in homozygous and heterozygous klotho gene deficient mice. We successfully bred homozygous klotho deficient mice through hybridization of heterozygous (klotho \(+/-\)) mice and tested ABR thresholds using clicks and tone-bursts in WT, heterozygous and homozygous klotho deficient mice. Comparing with the ABR threshold in WT and heterozygous (klotho \(+/-\)) mice, we found that homozygous klotho deficient mice showed more than 50 dB threshold shifts. Moreover, we also revealed that klotho gene deficiency caused more hearing loss at 4–16 kHz in mice.

2. Methods

2.1. Klotho deficient mice models

Heterozygous klotho deficient mice were provided by Dr. Gu Jun’s laboratory at Beijing University (Liu et al., 2011). In our laboratory, we hybridized klotho \(+/-\) mice and identified the gene type for each new-born mouse by PCR. The primer for genotyping are as follow: KL1: TGGAGATTGGAAGTGGACG; KL2: CAAGGACCAGTTCATCATCG, KL3: TTAAGGACTCCTGCATCTGC These mice were raised to 25 days old for genotyping and labeled. The size and weight of klotho \(-/-\) and WT mice at 30 days after birth (P-30) were recorded. Finally, click and tone burst ABRs were tested in wild type, heterozygous and homozygous klotho deficient mice at P-60.

2.2. Genotyping of wild type and klotho deficient mice

The cross-bred mice (raised by the Institute of Hearing and Balance Research at Xuzhou Medical University) were toe-tagged 25 days after birth. The toes were cut off and kept in 1.5ml EP tubes in which 200 µl tissue lysis buffer was added to extract whole genome DNA (in compliance with the protocol of DNA extraction kit). The density of the extracted DNA was subsequently measured while 50–300 ng genome DNA was used as a template for the amplification of special-site genome DNA fragments. Agarose gel (10%) electrophoresis was introduced to identify genes in klotho \(+/-\) mice based DNA bands.

2.3. Auditory brainstem response test

Mice (P-60) were anesthetized with ketamine (75 mg/kg)/xylazine (100 mg/kg). ABR responses were elicited by clicks and tone bursts at 4, 8, 16 and 32 kHz at increasing sound intensities ranging from 10 to 90 dB SPL in 10 dB steps. Tone bursts (5 ms duration and 0.5 ms rise/fall time) were presented at 51.1/second. Evoked potentials were averaged (n = 512–1024), amplified (Dagan 2400A preamplifier), filtered (100–3000 Hz), digitized (HEKA Elektronik), and stored on a computer. Thresholds, the lowest

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Fig. 1. The klotho knock-out mice model. (A) The mortality curve indicating shorter life time for klotho \(-/-\) (blue line) as compared with WT (red line) mice. Black arrow heads mark important experimental events, including genotyping (P-25), weighting and phenotyping (P-30) and ABR testing (P-60). klotho \(-/-\) mice typically die between P-65 and P-75. (B) Phenotype observation for WT (klotho \(+/+\)) and klotho \(-/-\) mice at P-30. (C) Weight analysis for WT and klotho \(-/-\) mice at P-30 (n = 4, *p < 0.05)
sound level that evoked a visible ABR, were determined for each tested frequency.

2.4. Statistical analysis

Statistical analysis was carried out using the SPSS 16.0 software (SPSS, USA). Data were expressed as mean ± standard error (SEM) and compared by Student’s t-test. Statistical significance level was set at P < 0.05.

3. Results

3.1. Phenotypes in WT and klotho deficient mice

In this mouse model, only heterozygous (klotho+/−) mice were used for breeding because the homozygous (klotho−/−) mice are infertile. The mortality curves showed that the life time of klotho−/− mice was significantly shorter than WT mice from the same litter (Fig. 1A). Other impotent time point information, including the time of genotyping, weighting, ABR test and the life time, is also labeled in Fig. 1A. The results showed that klotho−/− mice growth was far slower (Fig. 1B) and their average weight significantly lower than wide-type mice in the same litter (Fig. 1C).

3.2. Click evoked ABRs in klotho deficiency mice

Click evoked ABRs in wild type (klotho+/+, n = 4), heterozygous (klotho+/−, n = 4) and homozygous (klotho−/−, n = 4) klotho deficient mice showed various degrees of hearing loss in both heterozygous (8 ears) and homozygous (8 ears) klotho deficient mice (Fig. 2) as compared to WT mice (8 ears). Worse hearing loss was seen in klotho−/− mice with a number of other aging related diseases.

Fig. 2. Click-evoked ABRs in WT and klotho deficient mice (n = 8, NS P > 0.05, *p < 0.05, **p < 0.01).

Fig. 3. ABRs elicited by tone-bursts in WT and klotho deficient mice (n = 8, NS P > 0.05, *p < 0.05, **p < 0.01).
3.3. Tone-bursts induced ABRs in heterozygous and homozygous klotho deficient mice

To further reveal the specific frequencies affected by klotho deficiency, tone-bursts ABR thresholds were obtained, which revealed significant thresholds elevation at 4, 16 and 32 kHz in homozygous klotho gene deficient mice compared to WT mice, while thresholds in heterozygous mice were higher than in WT mice but lower than in homozygous klotho deficient mice (Fig. 3).

4. Discussion

In summary, our experiment confirms that klotho gene deficiency causes systemic abnormalities (Fig. 1) and profound hearing disorders (Figs. 2 and 3). We further elucidate that the hearing impairment is frequency specific in both heterozygous and homozygous klotho deficient mice. We also found that ABR thresholds at 16 and 32 kHz were seriously impaired, corresponding to the dosage effect of the klotho gene (Fig. 3) which have not been reported in previous studies (Kuroo et al., 1998; Kamemori et al., 2002; Takumida et al., 2009).

The hearing loss shown in these klotho gene deficient mice is similar with ARHL, a common hearing disease in the elderly population. Our study shows mild hearing loss in heterozygous klotho deficient mice and profound hearing loss in homozygous klotho deficient mice at P-25. These results indicate that the klotho gene is critical in maintaining normal hearing sensitivity. We postulate that constitutive expression of the anti-aging protein klotho in the inner ear may protect the sensorineural hearing system from ARHL. More studies are needed to test this hypothesis and find the link between the klotho gene and ARHL.

Conflicts of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

The klotho deficient mice were provided by Professor Ju Gu from Peking University. This work was supported by the National Nature Science Foundation of China (81470684, 21405130), Postdoctoral Science Foundation of China (2015MS571818), Clinical Special Fund of Jiangsu Province (B12014032), Six Major Categories Talent (2014-WSN-043, 2011-WS-074), Jiangsu Provincial University Fund (16621632), Innovation and Entrepreneurship Training Program for College Students in Jiangsu Province (KYLX14-1455, 201610313002Z), Colleges and universities Foundation in Jiangsu Province (16621632, 16KJ8320016). Nature Science planning Foundation of Xuzhou (KCI7087).

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