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Reduced expression of citrate synthase leads to excessive superoxide formation and cell apoptosis

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ABSTRACT

A/J mice are a mouse model of age-related hearing loss. It has been demonstrated that a mutation in gene of *citrate synthase* (CS) contributes to the early onset of hearing loss occurring at about one month of age. To understand the effects of a decreased CS activity that results from the mutation in *Cs* gene on hearing loss in A/J mice, human kidney cell line (293T) was transiently transfected with short hairpin RNA for *Cs* (shRNA-Cs) to reduce expression of CS. In comparison with those of cells transfected with a scrambled sequence (shRNA-NC), the oxygen consumption rate and adenosine trisphosphate (ATP) production level were decreased in 293T cells transfected with shRNA-Cs. Meanwhile, excessive superoxide production was induced as determined by mitochondrial superoxide formation assay (MitoSOX) and superoxide dismutase 2 (SOD2) detection. Moreover, the expression levels of BIP (binding immunoglobulin protein) and CHOP (CCAAT/enhancer-binding protein-homologous protein), markers of endoplasmic reticulum stress, were upregulated. Furthermore, apoptosis related molecule caspase-3 and the mitochondrial membrane potential were reduced. It is therefore concluded that downregulation of *Cs* expression in 293T cells leads to low level of ATP production, excessive superoxide formation and cell apoptosis, which implies a possible mechanism for hearing loss in A/J mice.

1. Introduction

Age-related hearing loss (AHL), or presbycusis, is a global concern of aging, and is the most common sensory defect in the elderly population. AHL refers to more than 40% of people over the age of 65 in the United States and is predicted that 28 million American will suffer from this disorder by 2030 [1]. The molecular mechanisms of the different types of AHL are not understood and there is as yet no known way to prevent or treat this disorder. A/J mice are a mouse model of age-related hearing loss [2,3]. It has been demonstrated that a mutation in citrate synthase (CS) gene, which is known as *ahl4* locus, can explain about 40% of the ABR threshold variation in the mice [2,4,5]. A/J mice differ in CS enzyme kinetics and catalytic properties from mice of other strains and, specifically, alteration of H55 N in CS caused 50%–65% reduction of CS activity in skeletal muscle of A/J mice [6]. Impaired citrate synthase activity could thus impact mitochondrial function and exacerbate AHL [4]. However, the detailed mechanism needs further investigation.

Citrate synthase (CS) is an essential enzyme and the first rate-limiting enzyme in the tricarboxylic acid cycle (TCA) that generates high-energy reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH)2 for oxidative phosphorylation [7]. CS migrates from the cytoplasm to mitochondrial matrix to exert its function [8]. An increasing number of studies have shown that CS plays a key role in maintaining energy production in different types of cells [9].

In a previous study, we showed that caspase-mediated apoptosis in the cochlea contributes to the early onset of hearing loss in A/J mice [3]. Therefore, we try to investigate the mechanism

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of cell apoptosis in the cochleae of A/J mice by downregulation the level of CS in mammalian cell line. We found that downregulation of *Cs* expression led to low level of ATP production, excessive superoxide formation and cell apoptosis. The findings in this study may aid in understanding the mechanism of hearing loss in A/J mice. Moreover, the CS related oxidative or apoptotic pathways may be the potential targets for AHL prevention and therapy.

2. Materials and methods

2.1. Designing shRNA and construction of pGPU6/Neo/homo-Cs

Short hairpin RNA (shRNA) sequences for human *Cs* gene were designed by software of siRNA (small interfering RNA) Sequence-Selector and synthesized in Shanghai Biotech, Ltd. Corp., China. The oligonucleotide sequences targeting human *Cs* gene, which are listed in Table 1, were inserted into *Bbs*I and *BamH*I sites of the pGPU6/Neo to generate pGPU6/Neo/homo-Cs. The recombinant plasmids were designated as pGPU6/Neo/Cs897, pGPU6/Neo/Cs1227, pGPU6/Neo/Cs1280 and pGPU6/Neo/Cs1408, respectively. pGPU6/Neo/NC containing a scrambled sequence (NC) of shRNA with no significant similarity to human gene was used as the negative control. pGPU6/Neo/Gapdh containing shRNA sequence that targets glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) of human was used as positive control.

2.2. Cell culture and transfection

293T cells (Kept in our laboratory) were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) containing 10% fetal bovine serum (Gibco) at 37 °C under permissive condition of 5% CO2. For transfection experiments, 293T cells were plated into 6-well plates (4×10^5 cells/ml) for 24 h to reach 50–70% confluency. The cells were then transfected with plasmids transcripting shRNA-Cs, shRNA-NC and shRNA-Gapdh using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 36 h post-transfection, 293T cells were collected for further experiments.

Table 1
Oligonucleotide sequences of human shRNA.

ID	Selected sequences	Template sequences
shRNA-Cs897	5′-GGTCTCACAATTTCACCAACA-3′	5′-CACCGGTCTCACAATTTCACCAACATTCAAGAGATGTTGGTGAAATTGTGAGACCTTTTTTG-3′
shRNA-Cs1227	5′-GCCATGCAGTACTAAGGAAGA-3′	5′-CACCGCCATGCAGTACTAAGGAAGATTCAAGAGATCTTCCTTAGTACTGCATGGCTTTTTTG-3′
shRNA-Cs1280	5′-GCTCTGAAACACCTGCCTAAT-3′	5′-CACCGCTCTGAAACACCTGCCTAATTTCAAGAGAATTAGGCAGGTGTTTCAGAGCTTTTTTG-3′
shRNA-Cs1408	5′-GGTGCTGCTCCAGTATTATGG-3′	5′-CACCGGTGCTGCTCCAGTATTATGGTTCAAGAGACCATAATACTGGAGCAGCACCTTTTTTG-3′
shRNA-Gapdh	5′-GTATGACAACAGCCTCAAG-3′	5′-CACCGTATGACAACAGCCTCAAGTTCAAGAGACTTGAGGCTGTTGTCATACTTTTTTG-3′
shRNA-NC	5′-GTTCTCCGAACGTGTCACGT-3′	5′-CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTG-3′

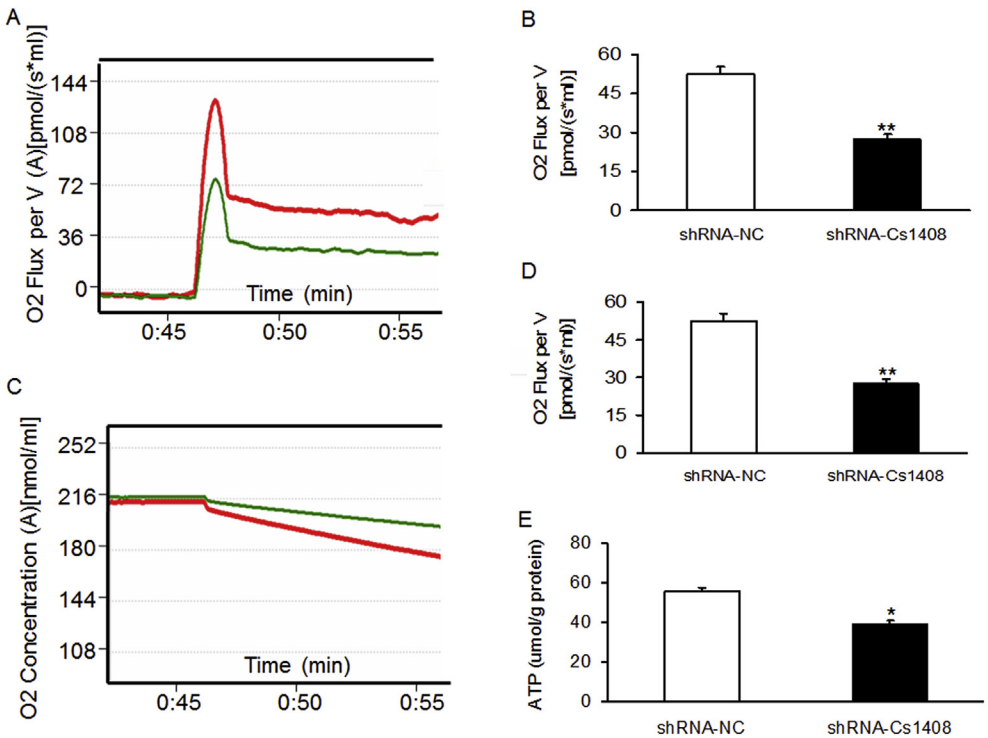

Fig. 1. Oxygen consumption rate and ATP production levels were reduced in 293T cells 36 h after transfection. (A) The oxygen consumption rate was tested by high-resolution respirometry in 293T cells transfected with shRNA-Cs1048 (green line) and shRNA-NC (red line). (B) Quantitative analysis of the results showed that oxygen consumption rate in Cs knockdown cells was reduced in comparison with that of the control. (C) Mean O_2 flux in the chamber of cells transfected with shRNACs1048 (green line) and that of the control (red line) 10 min after loading of cells. (D) O_2 concentration in the chamber of *Cs* knockdown cells was high in comparison with that of the control 10 min after loading of cells. (E) Downregulation of *Cs* expression reduced ATP generation of in 293T cells. ATP levels in the cells were measured 36 h post-transfection. The levels of ATP in Cs knockdown cells were significantly decreased in comparison with that in the control. All data presented as mean ± SEM of triplicate experiments. *$P < 0.05$, **$P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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