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Research article

Insulin resistance due to dietary iron overload disrupts inner hair cell ribbon synapse plasticity in male mice

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HIGHLIGHTS

- We use an insulin resistance model in C57Bl/6 male mice by using an iron-enriched diet.
- Insulin resistance due to dietary iron overload may cause a moderate hearing loss.
- Inner hair cells ribbon synapses may be more susceptible to insulin resistance due to dietary iron overload.

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ABSTRACT

To evaluate whether cochlear inner hair cells (IHCs) ribbon synapse plasticity would be interrupted by insulin resistance (IR) due to dietary iron overload, we established an IR model in C57Bl/6 male mice with an iron-enriched diet for 16 weeks. Glucose levels were measured at weeks 4, 8, 12, 16. Glucose tolerance test and insulin tolerance test were performed at week 16 after overnight fasting. Then, auditory brainstem responses (ABRs) measurements were performed for hearing threshold shifts. After ABR measurements, cochleae were harvested for assessment of the number of IHC ribbon synapses by immunostaining, the morphology of cochlear hair cells and spiral ganglion neurons (SGNs) by transmission electron microscopy or immunostaining. Here, we show that IR due to dietary iron overload decreased the number of ribbon synapses, and induced moderate ABR threshold elevations. Besides, additional components including outer hair cells (OHCs), IHCs, and SGNs were unaffected. Moreover, IR did not disrupt the expression of vesicular glutamate transporter 3 (VGLUT3), myosin VIIa and prestin in hair cells. These results indicate that IHC ribbon synapses may be more susceptible to IR due to dietary iron overload.

1. Introduction

Iron overload is an independent factor contributing to the development of type 2 diabetes mellitus (DM2), which may lead to insulin resistance (IR) and reduction in insulin secretion [1]. Higher body iron stores and higher heme iron intake are associated with a greater risk of DM2 [2]. Recently, epidemiological evidence from a systematic review and meta-analysis has suggested a modest association between DM2 and alterations in hearing [3]. Moreover, previous animal studies have explored that DM2 caused degeneration of outer hair cells (OHCs) [4,5] and inner hair cells (IHCs) [4], loss of spiral ganglion neurons (SGNs) [6]. Hearing impairment is related to abnormal processing of the spectrotemporal properties of sounds all along the auditory pathway [7]. In mammals, the performance of auditory pathway involves the unique properties of the IHCs, which are functionally coupled to the OHCs to improve sound detection [8]. Moreover, the auditory pathway must operate precisely in order for acoustic signals to be encoded and transmitted to the central nervous system [7]. Auditory brainstem response (ABR) is a neurological screening test to assess the integrity of the peripheral auditory nerve and the lower part of the brain [9]. In general, hearing relies on the precise and rapid synaptic transmission at ribbon synapse [10]. These genuine sensory cells of the cochlea encode acoustic signals by graded variations of their membrane potential trigger rapid and sustained vesicle exocytosis at their ribbon synapses [8]. Thus, development

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and function of IHC ribbon synapse is pivotal to auditory and speech perception. However, little is known about whether IHC ribbon synapses can be regulated by dietary iron overload. Furthermore, it is unclear if there are implications for ribbon synapse plasticity in response to dietary iron overload. Also, we do not know about whether changes in ribbon synapse occur prior to alterations of cochlear hair cells and SGNs.

Ribbon synapse is the first afferent neuronal connection in the auditory pathway, and formed between IHC and SGN [11]. Besides each SGN receives input from only one IHC synapse [12]. RIBEYE is the major structural component of synaptic ribbons [13], and a specific protein prominently expressed in ribbon synapse containing neurons such as retinal photoreceptors and bipolar cells, pinealocytes of epiphysis or hair cells of cochlea [14]. RIBEYE has a B-domain that is identical with transcriptional repressor carboxyterminal binding protein 2 (CtbP2), which produced virtually identical staining patterns [14]. In fact, RIBEYE/CtbP2 is widely recognized as a marker for presynaptic component of ribbon synapse. Between IHC and SGN, glutamate-mediated synaptic transmission needs vesicular glutamate transporters (VGLUTs) load glutamate into the synaptic vesicles [15]. Specifically, VGLUT3 is the most important type among the three VGLUT isoforms in the cochlea [16]. Likewise, myosin VIIa is a special protein prominently expressed in sensory hair cells, and plays a pivotal role in vesicle cycling of cochlear hair cells [17]. Consequently, the present study was designed to test the effects of IR due to dietary iron overload on ribbon synapse plasticity between IHC and SGN.

2. Methods

Sixteen C57Bl/6 mice (20 ± 1 g; male) were obtained from the Center for Experimental Animals at China Medical University (Shenyang, China) with a normal Preyer's reflex. For the randomization procedure, the mice were weighed and ranked according to body weight. These mice were assigned to one of two dietary groups: One group (control group; $n = 8$) was fed a normal diet (iron content: 8.26 ± 2.67 mg/kg diet, measured by atomic absorption spectrometry), and following the AIN-93G purified rodent diet guidelines [18]. The other group (iron-enriched diet group; $n = 8$) was fed an iron-enriched diet (IED, carbonyl-iron) for a period of 16 weeks. This time point (16 weeks used as the observation period) was mainly based on the previous report to induce IR by dietary iron overload [19]. Iron content of IED was detected at 8.39 ± 1.03 g/kg feed. The dose of carbonyl-iron was based on prior study [19]. Venous blood glucose levels were measured at baseline (pretreatment) and 4, 8, 12 and 16 weeks after fed IED or standard diet. ABR measurements, glucose tolerance test (GTT; 2 g/kg of glucose) and insulin tolerance test (ITT; 0.5 U/kg) were performed after prolonged overnight fasting at 16 weeks. The ABR test was a modification described [20]. GTT and ITT were performed as previously described [21].

The ultrastructure of stereocilia of IHCs was observed by transmission electron microscope following a standardized analytical method [20]. The cochlea was removed following a standardized method [7]. The organ of Corti was post-fixed with 1% osmium tetroxide and 0.01% potassium dichromate for 2 h at room temperature, dehydrated in graded ethanol solutions from 50% to 100% (each for 30 min) and then 100% acetone (three changes, each for 10 min). The samples were immersed in a mixture of 50% epoxy resin, 50% pure acetone for 30 min at room temperature. Each sample was placed on a Teflon support, covered with a capsule containing pure epoxy resin for 1 h at 60 °C and polymerised for 24 h at 80 °C. After the samples were trimmed, serial sections (70 μm) were collected on copper grids and stained with uranyl acetate, followed by lead citrate.

Table 1
Characterization of mice fed either control or IED after 16 weeks.

Group	Control	IED
SI (μmol/l)[a]	46.07 ± 6.82	64.34 ± 9.01*
Hb (g/l)[a]	128.50 ± 11.93	135.46 ± 14.63
TIBC (μmol/l)[a]	88.16 ± 6.38	90.72 ± 7.12
Transferrin (g/l)	3.56 ± 0.13	3.59 ± 0.72
TS (%)	52.08 ± 5.91	75.72 ± 6.12*
SF (ng/ml)	76.04 ± 7.22	166.03 ± 9.16*
FPG (mmol/l)	4.0 ± 0.1	4.1 ± 0.1
FPI (pmol/l)	126.0 ± 13.9	231 ± 20.7*
HOMA-IR	0.5 ± 0.0	0.89 ± 0.43*

FPG, fasting plasma glucose; FPI, fasting plasma insulin; Hb, Hemoglobin; HOMA-IR, insulin resistance index; SF, serum ferritin, SI, serum level of iron; TIBC, total iron binding capacity; TS, transferrin saturation.

* $P < 0.05$ versus control group.

[a] Mean ± SEM.

The assessments of histology were performed as previously described [7]. The separated basilar membrane were fixed in 4% paraformaldehyde and dissolved in 0.1 M PBS with 30% sucrose for 1 h at room temperature. Next, the samples were washed three times in 0.01 M PBS and preincubated for 30 min at room temperature in blocking solution of 5% normal goat serum in 0.01 M PBS with 0.3% Triton X-100, then were incubated with mouse anti-CtBP2 (1:100, BD), rabbit anti-myosin VIIa (1:200, Santa Cruz), rabbit anti-VGLUT3 (1:200, ABCam) or goat anti-prestin (1:100, Sigma) left 4 °C for 24 h. Then, the incubated samples were washed out in 0.01 M PBS for three times, and incubated with the secondary antibody Alexa Fluor 488 goat anti mouse IgG (1:200, Invitrogen), Alexa Fluor 568 goat anti rabbit IgG (1:200, Invitrogen) or Alexa Fluor 488 donkey anti goat IgG (1:200, Invitrogen) at 37 °C for 2 h. After incubation, the samples were washed in PBS twice. Dropping a drop of DAPI (4′,6-diamidino-2-phenylindole; Santa Cruz) in the slide, basement membrane were tiled under a dissecting microscope, the coverslip covered the slide.

All analyses were carried out using SPSS software (version 12.0; SPSS Inc Chicago, IL). Data are presented as mean ± SEM and considered statistically significant at $P < 0.05$. Student's t-test and Chi square test were used to analyze the group difference.

3. Results

3.1. Body weight, iron parameters and blood glucose assays

Body weight increased steadily in control group throughout the 16-week observation period. In contrast, C57Bl/6 mice fed IED exhibited little gain in body weight. Body weights of the mice fed IED were significantly decreased compared with those of controls at 4, 8, 12 and 16 weeks ($P < 0.05$) (Fig. 1A) despite similar food intake (control: 3.43 ± 0.60 g/day per mouse, IED: 3.50 ± 0.70 g/day, per mouse). SI and TS of mice fed IED were significantly increased compared with those of controls (Table 1, $P < 0.05$). The blood glucose levels of mice fed IED were significantly higher compared with those of controls after 8 weeks (Fig. 1B, $P < 0.05$), and the difference was maintained during the treatment period. Glucose levels of mice fed IED remained higher at the end of the GTT, but there was a lower increase during the first phase (Fig. 1C). Insulin resistance was confirmed by ITT, demonstrating increased glucose levels of mice fed IED at each time point compared with those of controls (Fig. 1D). Furthermore, these mice fed IED showed higher HOMA-IR index than those of controls (Table 1).

3.2. ABR threshold shift and cochlear hair cells

The ABR threshold elevations of mice fed IED were slightly higher compared with those of controls, but these differences did