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Increased sensitivity of apolipoprotein E knockout mice to copper-induced oxidative injury to the liver

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ABSTRACT

Apolipoprotein E (ApoE) genotypes are related to clinical presentations in patients with Wilson's disease, indicating that ApoE may play an important role in the disease. However, our understanding of the role of ApoE in Wilson's disease is limited. High copper concentration in Wilson's disease induces excessive generation of free oxygen radicals. Meanwhile, ApoE proteins possess antioxidant effects. We therefore determined whether copper-induced oxidative damage differ in the liver of wild-type and ApoE knockout ($ApoE^{-/-}$) mice. Both wild-type and $ApoE^{-/-}$ mice were intragastrically administered with 0.2 mL of copper sulfate pentahydrate (200 mg/kg; a total dose of 4 mg/d) or the same volume of saline daily for 12 weeks, respectively. Copper and oxidative stress markers in the liver tissue and in the serum were assessed. Our results showed that, compared with the wild-type mice administered with copper, TBARS as a marker of lipid peroxidation, the expression of oxygenase-1 (HO-1), NAD(P)H dehydrogenase, and quinone 1 (NQO1) significantly increased in the $ApoE^{-/-}$ mice administered with copper, meanwhile superoxide dismutase (SOD) activity significantly decreased. Thus, it is concluded that ApoE may protect the liver from copper-induced oxidative damage in Wilson's disease.

1. Introduction

Wilson's disease (WD) is an autosomal recessive disease caused by mutations in the ATP7B gene, and can result in copper metabolism disorders [1]. The incidence of WD is approximately 1 in 30,000 live births [2]. In European populations, the most common mutation in patients with Wilson's disease is the H1069Q mutation, however, the R778L mutation accounts for the most in Asian populations [2–5]. Patients with WD exhibit various clinical manifestations, such as different degrees of hepatic damage, neuropsychiatric symptoms, Kayser–Flescher rings, and damage to the kidney and skeletal muscle [6]. The high variability of clinical manifestations of WD patients can partially be due to different types of ATP7B gene mutations; genetic polymorphisms in the methyltetrahydrofolate reductase gene, cytokine genes, and prion-related protein gene; as well as influence of the patient's sex. The ApoE gene has three alleles named as ε2, ε3, and ε4, which encode three ApoE isoforms with different biological functions [10]. Considerable evidence indicates that ApoE genotype is associated with Alzheimer's disease, vascular diseases, and brain injury [10–14]. The ApoE ε3 allele is believed to be neuroprotective, whereas the ApoE ε4 allele exacerbates neuronal damage in Alzheimer's disease [15–17]. There is a documented association between ApoE genotype and WD clinical expression [7–9]. However, the mechanisms by which ApoE proteins are involved in WD remain unknown. ApoE genotype might affect WD by isoform-specific effects of ApoE on copper-induced oxidative tissue injury. It has been reported that the ApoE protein isoform less effectively protects neurons from oxidative-damage [18]. There is not yet a definite conclusion on whether ApoE has an antioxidant effect in Wilson's disease.

We aimed to determine the antioxidant effect of ApoE in Wilson's disease. In the present study, we established an animal model of Wilson's disease by feeding C57BL/6 mice and $ApoE^{-/-}$ mice with copper, and compared oxidative damage to the liver in the two strains of mice.

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2. Materials and methods

2.1. Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University. Male wild-type and ApoE-/- C57BL/6 mice (weighing 15.1 ± 1.45 g) were used in this study. The animals were obtained from the Animal Care Center of Beijing Medical University, China. Prior to the experiments, the animals were housed in the animal room of our institute at room temperature (23 ± 1 °C) with 60% humidity. Four mice were housed per cage on a 12 h light/dark cycle (lights on 08:30–20:30). Mice had free access to food and water. After acclimatization to the experimental conditions, the mice were randomly assigned to four groups ($n = 12$ per group): wild-type mice treated with saline(WT), ApoE$^{-/-}$ mice treated with saline(ApoE), wild-type mice treated with copper, and ApoE$^{-/-}$ mice treated with copper. Wild-type or ApoE$^{-/-}$ mice were intragastrically administered with 0.2 mL of copper sulfate pentahydrate (200 mg/kg; a total dose of 4 mg/d; lot # BCBG7381V, Sigma, USA) or the same volume of saline daily for 12 weeks, respectively. Animals were sacrificed, and blood samples were collected from the eyes. The liver, kidney and brain was removed and stored at −80 °C for the following experiments.

2.2. Preparation of tissue samples

To obtain serum, the blood samples were centrifuged at 3,000 rpm/min at room temperature for 20 min. Plasma was collected in 0.2-mL plastic tubes and diluted with deionized water in a ratio of 1:10. Serum was used for the measurement of copper, TBARS, and SOD.

To obtain liver, kidney or brain tissue homogenates, the liver or brain were homogenized in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Sigma–Aldrich, USA) using a homogenizer. Homogenates were centrifuged at 8,000 *g* for 10 min to obtain the supernatant. The supernatant was used for the determination of TBARS and SOD activities.

2.3. Measurement of copper levels

To measure copper concentrations in the liver or brain, liver or brain samples (150–200 mg) were digested with concentrated nitric acid (5 mL) and 30% H_2O_2 (1 mL) at 120 °C for 2 min, 160 °C for 2 min, and 180 °C for 5 min, using a microwave digestion system (CEM, USA). After the samples were cooled down, they were heated to 140 °C until the volume declined to 2 mL. Each sample was then washed with 1% nitric acid three times. Copper concentrations in the serum, liver, kidney, and brain were measured using an atomic absorption spectrometer (Beijing Purkinje General Instrument Co., Ltd., China).

2.4. Determination of TBARS and SOD

TBARS were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). Briefly, liver homogenates were mixed with 1 mL of TBA. The mixture was incubated at 95 °C for 40 min. The samples were then centrifuged at 4000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was expressed as nanomoles of TBARS per milligram of protein.

SOD was determined using the xanthine oxidase method with commercial kits (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer's instructions. Tissue homogenates were mixed with reaction solution, and the mixture was incubated at 37 °C for 30 min. The absorbance was measured at 450 nm. SOD activities were expressed as units per milligram of protein.

2.5. Western blot

Liver tissue proteins were prepared using RIPA protein extraction reagent (Sinoble, Beijing, China), according to the manufacturer's instructions. The tissues were homogenized on ice in lysis buffer. Equal amounts of proteins were separated by electrophoresis in 10% SDS–PAGE and transferred onto polyvinylidene fluoride membranes by electroblotting. The membranes were blocked with 3% bovine serum albumin in tris-buffered saline–Tween 20 and then were incubated with primary antibodies against NQO1 (dilution 1:2000; rabbit anti-mouse monoclonal antibody, Epitomics), HO-1 (dilution 1:2000; rabbit anti-mouse monoclonal antibody, Epitomics), or GAPDH (dilution 1:20,000; rabbit anti-mouse monoclonal antibody, Beijing TDY Biotech Co., Ltd.) at 4 °C overnight. Blots were developed using horseradish peroxidase-linked secondary antibodies and a chemiluminescence detection system.

2.6. Statistical analysis

Data were expressed as mean ± standard SD. Two-way ANOVA was used to determine any interaction between the genotype (C57BL/6 and ApoE$^{-/-}$) and the treatment (saline and copper). If the interaction was significant, a least squares mean test was used for pairwise comparison. All statistical analysis was performed with SPSS 13.0 software and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Copper concentrations in the liver, brain, kidney, and serum

As shown in Fig. 1, compared with the mice treated with saline, the copper levels in the liver, kidney and brain were all significantly higher in both the wild-type mice and the ApoE$^{-/-}$ mice administered with copper ($P < 0.01$). Interestingly, compared with wild-type mice fed with copper, the copper levels in the kidney and brain, especially in the liver, were significantly higher in the ApoE$^{-/-}$ mice administered with copper ($P < 0.01$). However, there was no significant difference in the serum copper levels among the four groups ($P > 0.05$).

3.2. TBARS, and superoxide dismutase (SOD) activities in the liver and serum

As shown in Fig. 2, in the two strains of mice administered with copper, the liver and serum TBARS activities both significantly increased ($P < 0.01$) and SOD activities both significantly decreased ($P < 0.01$), as respectively compared to the same strain of mice treated with saline. In addition, compared with the wild-type mice treated with copper, the liver and serum TBARS activities were significantly higher ($P < 0.01$), and SOD activities were significantly lower ($P < 0.01$) in the ApoE$^{-/-}$ mice administered with copper.

3.3. The expression of heme oxygenase-1 (HO-1) and NAD (P) H dehydrogenase, quinone 1 (NQO1) in the liver

As shown in Fig. 3, the western blot results showed that, in the two strains of mice administered with copper, the expressions of HO-1 ($P < 0.01$) and NQO1 ($P < 0.01$) in the liver both significantly increased ($P < 0.01$) as respectively compared to the same strain of mice treated with saline. In addition, compared with the wild-type mice treated with copper, the expression of HO-1 and NQO1 in the

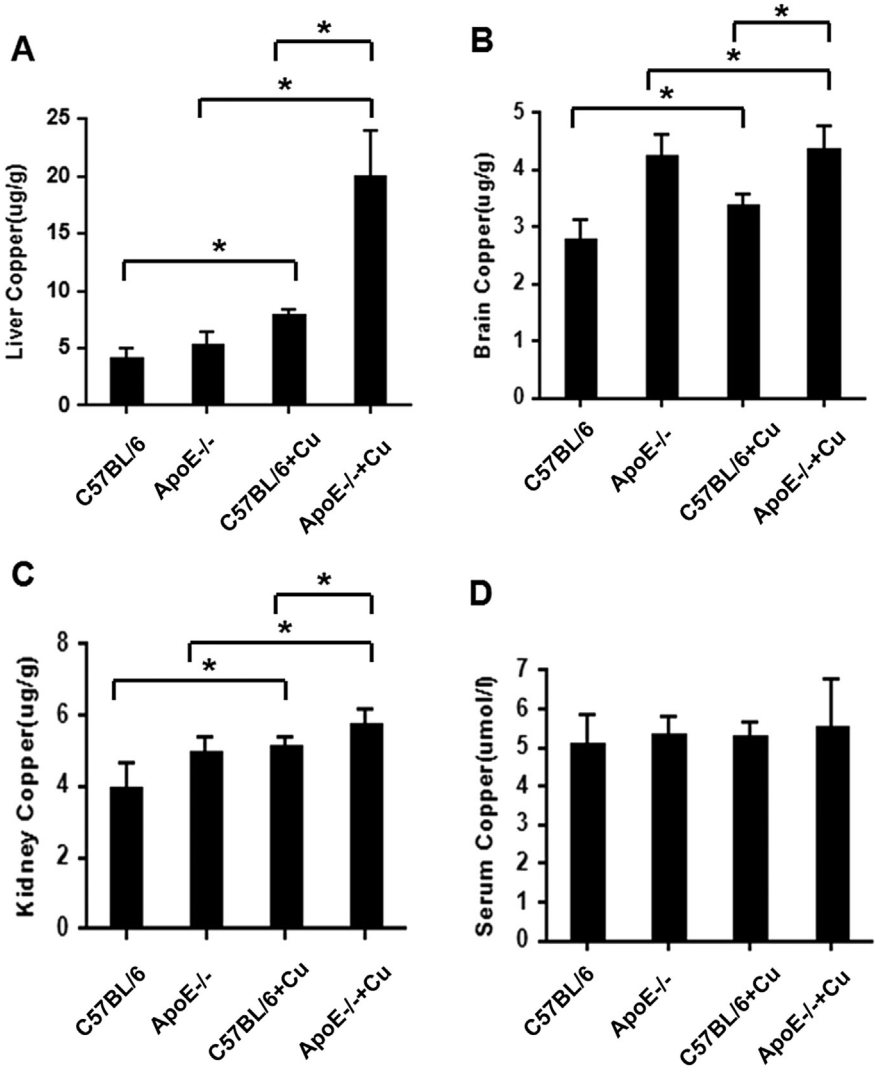

Fig. 1. Copper levels in the liver (A), brain (B), kidney (C), and serum (D). The 'C57BL/6', 'ApoE$^{-/-}$', ' C57BL/6 + Cu', and ' ApoE$^{-/-}$+ Cu ' represent wild-type mice administered with saline, ApoE$^{-/-}$ mice administered with saline, wild-type mice administered with copper, and ApoE$^{-/-}$ mice administered with copper. Values are expressed as mean ± SD (*$P < 0.01$).

liver were significantly higher in the ApoE-/- mice treated with copper ($P < 0.05$).

4. Discussion

In the present study, we investigated the effect of ApoE on copper-induced oxidative toxicity in the liver in ApoE-deficient mice treated with copper. We found that copper administration for 12 weeks resulted in oxidative damage in the liver in wild-type mice, showing an increase in TBARS activities, a decrease in SOD activities, and an upregulation of NQO1 and HO-1. ApoE-deficient mice suffered from more serious copper-induced oxidative damage to the liver.

Wilson's disease, caused by a defect in copper metabolism, can leads to copper accumulation in the liver. Although copper is an essential trace element involved in many physiological processes, excessive copper is toxic. In agreement with the clinical features of patients with Wilson's disease, animals intragastically adminstrated with copper exhibits excessive copper accumulation in the liver, kidney, and brain [19]. In toxicologic studies, copper is usually administered in doses of 50 mg/kg•d as low toxicity, 100 mg/kg•d as moderate toxicity, and 200 mg/kg•d as high toxicity [20]. We chose the dose of 200 mg/kg•d to ensure effectiveness of copper toxicity. On the other hand, it has been reported that, hepatic injury might occur after a period of 12 weeks feeding with standard chow diet in ApoE$^{-/-}$ mice [21]. In light of the above, 12 weeks of copper in high dosage was chose. In the present study, the copper level in the liver significantly increased, indicating that the intervention was effective.

Copper is necessary for the catalytic activity of many key enzymes involved in many cellular processes such as oxidative stress and respiration. Therefore, it is possible that multiple mechanisms contribute to copper-induced hepatic toxicity. It has been reported that copper can affect proliferation and viability of hepatic cells *in vitro* [22]. Additionally, copper-induced apoptosis via activation of acid sphingomyelinase can result in hepatic toxicity [23]. On the other hand, free copper can promote the generation of highly reactive oxygen radicals. It is resonable to believe that copper-induced hepatic toxicity could partly be due to copper-induced oxidative stress [24]. In agreement with the opinion, we found that TBARS activities and the expressions of NQO1 and HO-1 in the liver significantly increased, and SOD activities significantly decreased in the C57BL/6 mice administered with copper for 12 weeks. Furthermore, knockout of ApoE, a known antioxidant,

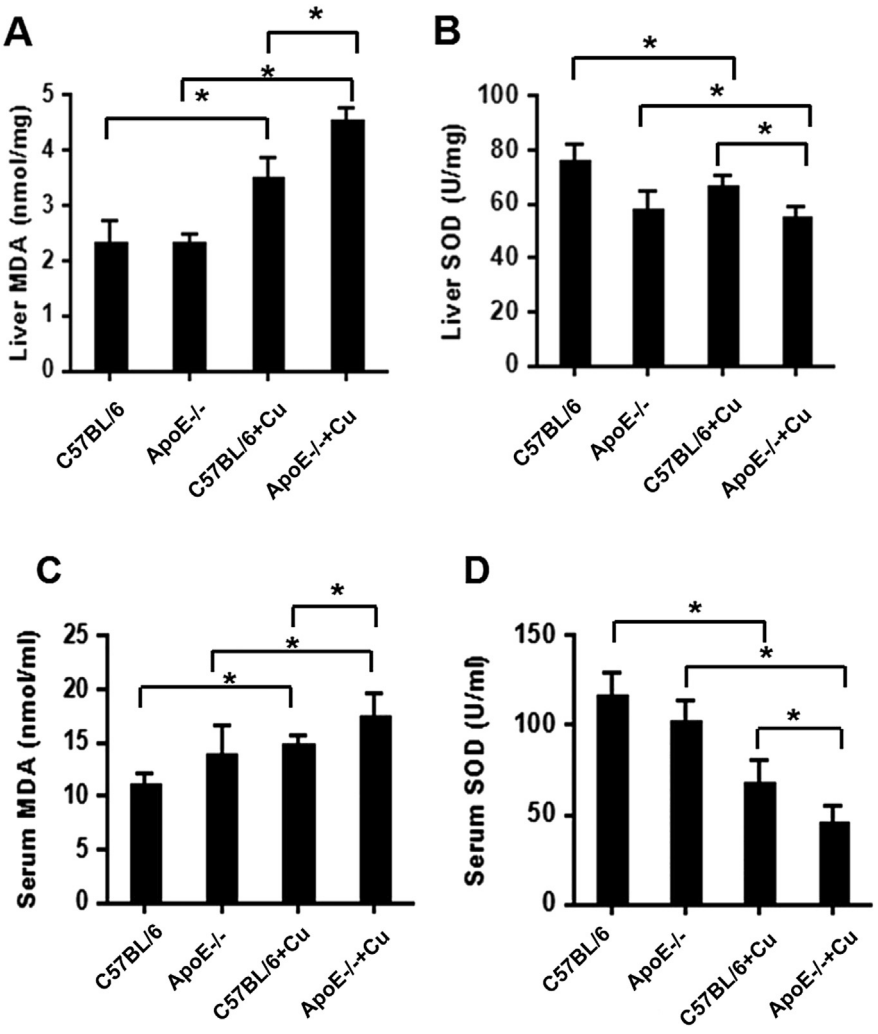

Fig. 2. The TBARS and SOD levels in the liver and serum. (A) The TBARS level in the liver. (B) The SOD level in the liver. (C) The TBARS level in the serum. (D) The SOD level in the serum. Values are expressed as mean ± SD(*$P < 0.01$).

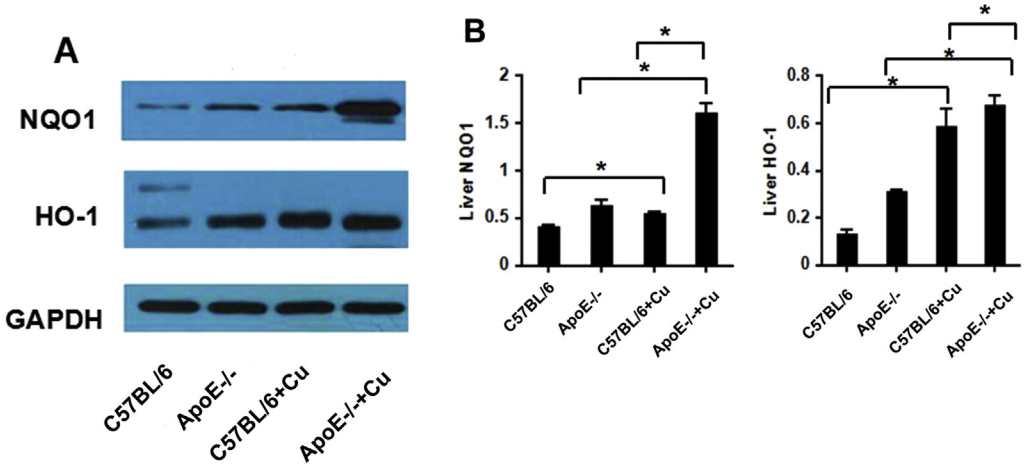

Fig. 3. Western blot analysis for NQO1 and HO-1 (A) protein level in the liver of four groups. Bar graphs illustrate the protein expression of NQO1 and HO-1 (B). Values are expressed as mean ± SD (*$P < 0.05$).

increased the copper-induced effects on TBARS, SOD, NQO1, and HO-1, further suggesting that copper-induced oxidative stress is involved in its hepatic toxicity.

ApoE genotypes have been found to be related with clinical outcomes of many diseases such as Alzheimer's disease, vascular disease, and brain injury [10,12–14]. In Alzheimer's disease, the ApoE 3 protein exerts a neuroprotective effect, whereas the ApoE 4 protein worsens neuronal injury [15–17]. Several studies have demonstrated that ApoE genotypes are associated with clinical presentations and age at onset of symptoms in patients with Wilson's disease [7–9]. However, it remains to be determined whether ApoE plays a protective role in Wilson's disease. In mouse, ApoE is of 70% homologous to human ApoE in the amino acid aspect [25]. In our study, $ApoE^{-/-}$ mice were used to investigate the possible role of ApoE in copper-induced oxidative injury in the liver. We found that, compared with wild-type mice administered with copper, $ApoE^{-/-}$ mice administered with copper exhibited higher TBARS, lower SOD activities, and higher expression levels of NQO1 and HO-1 in the liver. The results suggest that $ApoE^{-/-}$ mice are more sensitive to copper-induced oxidative damage, and ApoE may protect the liver from copper-induced oxidative injury.

Conflicts of interest

The authors declare no conflict of interest.

Transparency document

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