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Research Article

Copper induces hepatocyte injury due to the endoplasmic reticulum stress in cultured cells and patients with Wilson disease

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ABSTRACT

Copper is an essential trace element, however, excess copper is harmful to human health. Excess copper-derived oxidants contribute to the progression of Wilson disease, and oxidative stress induces accumulation of abnormal proteins. It is known that the endoplasmic reticulum (ER) plays an important role in proper protein folding, and that accumulation of misfolded proteins disturbs ER homeostasis resulting in ER stress. However, copper-induced ER homeostasis disturbance has not been fully clarified. We treated human hepatoma cell line (Huh7) and immortalized-human hepatocyte cell line (OUMS29) with copper and chemical chaperones, including 4-phenylbutyrate and ursodeoxycholic acid. We examined copper-induced oxidative stress, ER stress and apoptosis by immunofluorescence microscopy and immunoblot analyses. Furthermore, we examined the effects of copper on carcinogenesis. Excess copper induced not only oxidative stress but also ER stress. Furthermore, excess copper induced DNA damage and reduced cell proliferation. Chemical chaperones reduced this copper-induced hepatotoxicity. Excess copper induced hepatotoxicity via ER stress. We also confirmed the abnormality of ultra-structure of the ER of hepatocytes in patients with Wilson disease. These findings show that ER stress plays a pivotal role in Wilson disease, and suggests that chemical chaperones may have beneficial effects in the treatment of Wilson disease.

1. Introduction

Copper is an essential trace element and is indispensable for human activity [1–3]. It is required for several cellular processes, including synthesis of ceruloplasmin, melanin production, cytochrome c oxidation, decomposition of superoxides and synthesis of collagens [1]. Therefore, Menkes disease, which is an X-linked genetic copper deficiency, is a life-threatening disease and most patients with Menkes disease die by the age of 3 or 4 [4]. On the other hand, excess copper deposition is also harmful for various cells. Wilson disease is an autosomal recessive genetic disorder characterized by the accumulation of copper in various organs. *ATP7B* has been cloned as a causative gene in Wilson disease [5–8]. It has been described that the gene product of ATP7B, which is copper-transporting ATPase, is localized at the trans-Golgi network in hepatocytes and functions as the copper transporter [9,10]. However, we have reported that ATP7B locates in the late endosomes of hepatocytes [11–15]. Although this ATP7B localization remains a matter of controversy, dysfunction of this transporter induces excess copper accumulation in the body, particularly in the liver and brain.

Free radicals generated by excess copper bind the amino acid residue of proteins and alter protein structure [1,16]. Oxidative stress induces the accumulation of abnormal proteins [17]. Thus, excess copper–derived oxidants are associated with hepatotoxicity in Wilson disease [18]. Endoplasmic Reticulum (ER), which is an intracellular membranous organelle, has a protein-folding capacity. Many liver diseases, including non-alcoholic fatty liver disease, viral hepatitis, and alcoholic liver disease, are known to induce the accumulation of abnormal proteins in the ER [19–22]. This accumulation of abnormal proteins disturbs ER homeostasis resulting in ER stress [17]. ER stress is associated with various diseases, but the link between Wilson disease and ER homeostasis

Abbreviations: ALLN, acetyl-leucyl-leucyl-norleucinal; ATF6, activating transcription factor 6; BCS, bathocuproine disulfonate; ER, endoplasmic reticulum; GO, glucose oxidase; HCC, hepatocellular carcinoma; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; IRE1α, inositol-requiring 1α; NAC, n-acetyl-l-cysteine; PARP, poly-ADP-ribose-polymerase; PBA, 4-phenylbutyrate; PBS, phosphate-buffered saline; p-eIF2α, phospho-α-subunit of eukaryotic initiation factor 2; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PIs, proteasome inhibitors; ROS, reactive oxygen species; UDCA, ursodeoxycholic acid; XBP1, X-box binding protein 1

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is not fully understood. The chemical chaperone, 4-phenylbutyrate (PBA), has been shown to reduce the accumulation of misfolded proteins in the ER in patients with cystic fibrosis or α1-antitrypsin deficiency [23]. Ursodeoxycholic acid (UDCA), another chemical chaperone, protects hepatocytes against oxidative injury due to an increase in glutathione and thiol-containing proteins [24]. Although these findings, along with a published case report, suggest that chemical chaperones may have potential beneficial effects in the treatment of Wilson disease [25], the detailed mechanisms have not been evaluated.

A previous report showed that oxidative stress and ER stress induce cellular apoptosis in several liver diseases [26]. However, the effects of excess copper on hepatic cultured cells have not been fully elucidated. We confirmed the ER abnormality by electron microscopy in patients with Wilson disease.

In Wilson disease, hepatobiliary malignancies are rather rare [27]. But it has been reported that Wilson disease was a risk factor of hepatocellular carcinoma (HCC) [28]. Furthermore, it is suggested that cell proliferation is inhibited in Wilson disease [2].

Zinc acetate has been commonly used to treat Wilson disease because it increases the generation of metallothionein, a free radical scavenger, and inhibits absorption of copper in the intestinal epithelia [29]. However, whether or not zinc has a direct cytoprotective effect on hepatocytes is still unclear.

We evaluated the effects of chemical chaperones and zinc acetate on copper-induced disruption of cell homeostasis in hepatocytes. We also evaluated copper treatment-mediated DNA damage and hepatocellular proliferation in the presence or absence of chemical chaperones or zinc acetate, because of the low frequency of hepatocellular carcinoma, and the reduction of hepatocellular proliferation associated with the poor prognosis of acute liver failure in Wilson disease.

2. Materials and methods

2.1. Cells cultures and regents

We used human hepatoma cell lines established from hepatocellular carcinoma cell line (Huh7), highly differentiated immortalized human hepatocyte cell line (OUMS29) [30] and human embryonic kidney 293 cell line (HEK293). HEK293 expresses both ATP7A and ATP7B [31]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Cells were maintained in a 37 °C incubator with 5% CO_2. The following materials were used: copper sulfate; copper acetate; ferrous sulfate; manganese sulfate (Nacalai Tesque, Inc, Kyoto, Japan); acetyl-leucyl-leucyl-norleucinal (ALLN) and epoxomicin as proteasome inhibitors (PIs) (Calbiochem, La Jolla, CA, USA); bathocuproine disulfonate (BCS) as a copper chelator; n-acetyl-l-cysteine (NAC); glucose oxidase (GO); PBA and UDCA (Sigma-Aldrich, St. Louis, MO, USA); and zinc acetate (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Copper sulfate (300 μM) or copper acetate (300 μM) was administered for 12 h, because copper administration for 300 μM and 12 h showed appropriate cytotoxic effect. Ferrous sulfate (1 mM) or manganese sulfate (1 mM) was also administered for 12 h. BCS (150 μM), ALLN (7.8 μM) and epoxomicin (0.2 μM) were co-administered for 12 h, followed by immunoblotting analysis and immunofluorescence staining. Our preliminary study demonstrated that these concentrations of PI treatment did not induce apoptosis (data not shown). In the experiments with PBA (5 mM), UDCA (100 μM), NAC (10 mM) and zinc acetate (200 μM), PBA and UDCA were added 12 h, and NAC and zinc acetate were added 1 and 2 h before copper treatment, respectively.

Antibodies to the following antigens were used: ubiquitin, phospho α-subunit of eukaryotic initiation factor 2α (p-eIF2α), poly-ADP-ribose-polymerase (PARP), cleaved-caspase 3 (Cell Signaling Technology, Danvers, MA, USA), X-box binding protein 1 (XBP1), p62 (SQSTM1/sequestome1) (Santa Cruz Biotechnology, CA, USA); γH2AX (p Ser 139) (Novus Biologicals LLC, Littleton, CO, USA); Ki67 (Dako, Tokyo, Japan) and actin (Sigma-Aldrich, St. Louis, MO, USA). We used the primary and secondary antibodies at 1:1000 dilutions.

2.2. Detection of reactive oxygen species (ROS)

We detected ROS using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), a molecular probe for detecting intracellular H_2O_2 and oxidative stress [32]. H_2DCFDA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescein that reacts with H_2O_2 to form a fluorescent compound. After treatment with copper and GO, 10 μM H_2DCFDA was added to the medium for 30 min, then the cells were examined under a confocal laser scanning microscope (LSM5 Pascal, Carl Zeiss Micro Imaging Inc; Jena, Germany).

2.3. Immunofluorescence staining

Cells were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min, followed by incubation with the primary antibodies (for cleaved-caspase 3 and Ki67) for 1 h, washing with PBS, then incubation with the secondary antibodies for 1 h. Nuclear staining was done using 2.5 μg/mL of propidium iodide (Wako Pure Chemical Industries). For double labeling staining, images were acquired sequentially using separate excitation wavelengths (488 nm and 543 nm) then merged.

2.4. Immunoblotting analysis

We homogenized cells in a lysis buffer composed of 0.187 M trishydroxymethylaminomethane-HCl (pH 6.8), 10% sodium dodecyl sulfate and 5 mM ethylene diamine tetra-acetic acid. Equal amounts of protein were separated by polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to polyvinylidene fluoride microporous membrane (Millipore Corporation, Billerica, MA, USA). The blots were blocked with 0.2% skim milk in PBS containing 0.1% Triton X-100 followed by incubation with the primary antibodies then secondary antibodies (horseradish peroxidase-linked sheep anti-mouse antibody and donkey anti-rabbit antibody; GE Healthcare, Buckinghamshire, UK). The blots were visualized using enhanced chemiluminescence (ECL Plus Western Blotting Detection Reagents; GE Healthcare, Buckinghamshire, UK). The expression of each protein was measured by Light capture (ATTO Corporation, Tokyo, Japan). All blotting data were derived from at least 3 independent experiments.

2.5. Transmission electron microscopy

Human liver specimens were obtained by liver biopsy for the diagnosis of Wilson disease from two patients at the University of Occupational and Environmental Health hospital (Kitakyushu, Japan). Part of the specimens were processed for the electron microscopic examination. This examination was approved by the Institutional Review Board of our university (H23-39).

Information of the patients was provided in Table 1. We diagnosed the patients as Wilson disease according to the scoring system proposed at the 8th international meeting on Wilson disease [33]. We could diagnose the patients as Wilson disease without genetic analysis of *ATP7B* in Table 1.

Liver tissue pieces were fixed with 2% glutaraldehyde, post-