Supporting Information for

Original article

Cytochrome P450 endoplasmic reticulum-associated degradation (ERAD): Therapeutic and pathophysiological implications.

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Materials & Methods

1.1. Animals and treatments

Chip-knockout mice were generated and maintained on a mixed genetic background of C57BL/6 and 129SvEv according to previous studies (Kim et al.[140], 2016; Dai et al.[245], 2003). Liver-specific *Gp78*-knockout mice (C57BL/6-background) were generated as previously described (Kwon et al.[246], 2019). Mice were fed a standard chow diet, provided with water *ad libitum*, and maintained under 12-h light/dark cycle. All animal experimental protocols were approved by the UCSF/Institutional Animal Care and Use Committee (CA, USA).

1.2. Primary culture of mouse hepatocytes

Hepatocytes were isolated from male mice (8-week old) by *in situ* liver perfusion with collagenase and purification by Percoll gradient centrifugation at the UCSF Liver Center Cell Biology Core as described previously (Han et al.[247], 2005). The freshly isolated hepatocytes were seeded on 60 mm Permanox plates (Thermo Fisher Scientific, Waltham, MA, USA) coated with type I collagen, and incubated at 37 ℃ for 6 h in William's E medium containing 2 mmol/L L-glutamine, penicillin–streptomycin, insulin-transferrin-selenium, 0.1% bovine serum albumin Fraction V, and 0.1 μmol/L dexamethasone (DEX). The cells were overlaid with Matrigel (Matrigel® Matrix, Corning Inc. New York, NY, USA), and were stabilized for 2 days. For CYP2E1 or 3a induction, isoniazid (INH, 1 mmol/L) or DEX (10 μmol/L), respectively, was added to the cells on the 3rd day, and cells were treated with fresh medium containing the P450 inducer daily for another 2 days. To monitor the relative fructose-elicited fat accumulation of WT and gp78 KO hepatocytes, fructose 10 or 20 mmol/L was concurrently added to the cultures for the last 3 days. To examine the role of CYP2E1 in fructose-induced fatty

liver, hepatocytes were first pre-treated with INH (1 mmol/L) for 12 h, and incubated with both INH (1 mmol/L) and fructose (20 mmol/L) for 3 days. On the 3rd day, cells were then treated with the CYP2E1 inhibitor, 4-methylpyrazole (4-MP; X mmol/L), for the last 2 h before harvesting.

1.3. Oil red O staining

Cultured hepatocytes were fixed with 10 % neutral buffered-formalin for 1 h. Cells were incubated with oil red O (180 mg/mL, Cat. No. O0625, Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in 60% (*v*/*v*) isopropanol for 5 min followed by the incubation of hematoxylin solution (Cat. No. HHS16, Sigma–Aldrich Corp.) for 1 min. Cells were washed with warm water, and the red lipid and blue nuclei were observed through a microscope (Microphot-FXA, Nikon Instruments Inc., Tokyo, Japan) equipped with a camera (SPOT Imaging Model 1.3.0., Diagnostic Instruments, Inc., Sterling Heights, MA, USA).

1.4. Western immunoblotting analyses

Cells were harvested and lysed by ultrasonic homogenizer (Omni-Ruptor 250, Omni International Inc., Kennesaw, GA, USA) in cell lysis buffer (Cat. No. 9803, Cell Signaling Technology, Inc. Danvers, MA). The centrifuged (14,000 × *g*, 4 ℃, 10 min) whole cell lysates (proteins 5–15 μg) were used for the immunoblotting (IB) analyses as previously described (Kim et al.[140], 2016). Primary antibodies against CYP2E1 and CYP3A were purified from rabbit and goat sera, respectively. Commercial primary antibodies used in this study were listed in Table S3.

1.5. Cytotoxicity test

Cytotoxicty was measured by quantifying the medium adenylate kinase released from the damaged cells using the ToxiLight™ bioassay kit (Lonza Ltd., Basel, Switzerland).

1.6. Hepatic triglyceride determination

Intracellular triglyceride (TG) level of cultured primary hepatocytes was determined by Triglyceride Colorimetric Assay Kit (Cat. No. 10010303, Cayman Chemical, Ann Arbor, MI, USA).

1.7. Free fatty acid determination

Free fatty acids in cell homogenates were monitored by Free Fatty Acid Quantitation Kit (Cat. No. MAK044, Sigma–Aldrich Corp.)

1.8. Determination of oxidative stress

Malondialdehyde (MDA), a product of lipid peroxidation, was quantified using the cell homogenates by a thiobarbituric acid reactive substances (TBARS) assay kit (Cat. No. 10009055, Cayman Chemical, Ann Arbor, MI, USA).

1.9. SILAC-like (SILL) proteomic analyses

^{13}C-Lysine specific labeled liver tissue (^{13}C atom > 97 %; Cat. No. 252923900) from 8-week-old male C57BL/6 mouse was purchased from Silantes (Munich, Germany). The liver tissue was homogenized in cell lysis buffer (Cell Signaling Technology), and the centrifuged (14,000 × *g*, 4 ℃, 10 min) supernatant (lysate) was mixed with an equal protein amount of liver lysate from WT or *Gp78*-KO mouse of the same age. The mixed lysates (10 μg proteins) were subjected to SDS-PAGE for in-gel digestion. Gels were stained with Coomassie blue, and each gel lane was sliced into 10 sections. The gel pieces were reduced and alkylated, and then the proteins were digested by trypsin (1 μg; Promega Corp., Madison, WI, USA) and lysyl endoproteinase C (Lys-C, 1 μg; Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight at 37 ℃. The digested peptide mixtures were desalted by C_{18}-Ziptips (Sigma–Aldrich Corp.), and resuspended in 0.1% formic acid. For LC–MS/MS analyses, peptides were injected into a nanoAcquity UPLC system (Waters, Milford, MA, USA) and separated by EASY-Spray column (75 μm × 15 cm column packed with 3 μm, 100 Å PepMap C18 resin; Thermo Fisher Scientific) following a 60-min solvent gradient program using 0.1% formic acid and 0.1% formic acid in acetonitrile as mobile phases A and B, respectively, at a flow rate of 300 nL/min. The separated peptides were analyzed by an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) under high-energy collisional dissociation (HCD) mode. Peak lists from the 10 gel fractions were extracted by PAVA, an in-house software of UCSF mass spectrometry facility, and pooled to search against the Swissprot mouse database (SwissProt.2015.12.1) using ProteinProspector version 5.19.1 (http://prospector.ucsf.edu/prospector/mshome.htm). Following parameters for ProteinProspector search were used. Tolerance for parent and fragment ions were 20 ppm and 25 ppm. Carbamidomethylation of cysteine and ^{13}C-lysine were set as a fixed modification and a variable modification, respectively. The peptide numbers that are uniquely recognized as mouse proteins were obtained from the program to compare the relative protein amounts of WT and gp78 livers.

See Tables 1 and 2 in separated supporting information file.

Table S3. Specific antibodies used in IB-analyses.

Antibody	Clonality	Catalog number	Manufacturer
ACC	Rabbit monoclonal	3676s	Cell Signaling Technology
ACC (pS79)	Rabbit polyclonal	3661s	Cell Signaling Technology
Akt	Mouse monoclonal	MAB2055	R&D Systems, Inc.
Akt (pS473)	Rabbit polyclonal	9271s	Cell Signaling Technology
Akt (pT308)	Mouse monoclonal	MAB7419	R&D Systems, Inc.
AMPKα	Rabbit monoclonal	5831s	Cell Signaling Technology
AMPKα (pT172)	Rabbit monoclonal	2535s	Cell Signaling Technology
FAS	Rabbit monoclonal	3180s	Cell Signaling Technology
FOXO1	Rabbit monoclonal,	2880s	Cell Signaling Technology
FOXO1 (pT24)/FOXO3a (pT32)/FOXO4 (pT28)	Rabbit monoclonal	2599s	Cell Signaling Technology
FOXO3a	Rabbit monoclonal	12829s	Cell Signaling Technology
GAPDH	Rabbit monoclonal	2118s	Cell Signaling Technology
Histon H3	Rabbit polyclonal	ab1791	Abcam, plc.
Insig-1	Rabbit polyclonal	3854-30T	BioVision, Inc.
Insig-2	Rabbit polyclonal	ab86415	Abcam, plc.
LKB1	Rabbit monoclonal	3047s	Cell Signaling Technology
LKB1 (pS428)	Rabbit monoclonal	3482s	Cell Signaling Technology
PGC-1α	Rabbit polyclonal	ab54481	Abcam, plc.
SCD-1	Rabbit monoclonal	MA5-14885	Thermo Fisher Scientific, Inc.
SREBP-1	Mouse monoclonal	sc-365513	Santa Cruz Biotechnology, Inc.

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