Inactivation of Sirtuin2 protects mice from acetaminophen-induced liver injury: possible involvement of ER stress and S6K1 activation

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Supplementary Experimental Procedures

**Animals**

Ten-week-old C57BL/6J male mice were purchased from Orient Bio (Sungnam, Korea) and Sirt2 knockout (Sirt2 KO) mice were generated as previously described (48). Sirt2 KO mice of C57BL/6 background were kindly provided by Dr. H. S. Kim (Ewha Womans University, Seoul, Korea) (48). The mice had free access to food and water and were maintained at a temperature of 23 ± 2 °C, a humidity of 60 ± 10%, and a 12-hour light/dark cycle. Prior to APAP and AGK2 administration, mice were fasted for 15 h but had free access to water. APAP was dissolved in warm distilled water (60 °C) and intraperitoneally injected (500 mg/kg) into overnight-fasted mice to induce hepatitis. AGK2 was dissolved in dimethyl sulfoxide (DMSO) and intraperitoneally injected (1 mg/kg) 2 h prior to APAP administration. At 6 and 12 h after injection, the mice were anesthetized and killed; blood was collected via cardiac puncture. Tissues were harvested and either snap frozen in liquid nitrogen and stored at −70 °C or fixed in formalin and embedded in paraffin. All animal studies were approved by
the Animal Care and Use Committee of the Yonsei University College of Medicine.

**Immunohistochemistry**

For immunohistochemistry, sample were fixed with 4% paraformaldehyde and embedded in paraffin. Next, 10-μm sections were deparaffinized and sequentially rehydrated. Antigen retrieval was performed using the Target Retrieval solution, pH 6 (S1699, DAKO, Glostrup, Denmark) in a pressure cooker for 15 min. After cooling on ice for at least 1 h, the sections were incubated in 3% H₂O₂ for 30 min to block endogenous peroxidase activity. The sections were washed two times with PBS and incubated with Protein block serum-free (DAKO, X0909) for 2 h at room temperature for reducing nonspecific signals. Following incubation with primary antibodies, overnight at 4 °C in the presence of 3-nitrotyrosine (Millipore, Daejeon, Korea), and 3 washes in PBS, the sections were incubated with horseradish peroxidase-conjugated rabbit secondary antibodies (DAKO, K4003) for 15 min at room temperature. For immunohistochemistry, 3,3'-diaminobenzidine (DAKO, K3468) was used for development and Mayer’s Haematoxylin (DAKO, S3309) was used for counterstaining.

**Statistical analysis**

Data in the graphs were analysed using a two-tailed Student’s t test for comparisons between 2 groups, or a one-way ANOVA with Tukey’s honest significant difference post hoc test for multiple comparisons (SPSS 12.0K for Windows, SPSS, Chicago, IL) to determine statistical significance. A value of P < 0.05 was considered significant.

**Biochemical analyses**

Serum levels of alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) were measured by ELISA (BioAssay Systems, Hayward, CA, USA).
**Histological analysis**

Liver tissue fixed with 4% buffered formalin solution was embedded in paraffin and sectioned. Sections (10 μm thick) were then depleted of paraffin, subjected to H&E staining, and examined in a blinded manner for grading the ballooning degeneration of hepatocytes. 0 = none, 1 = few balloon cells, 2 = many cells/prominent ballooning (48). The liver necrotic area was diagnosed by a pathologist (K.T.N) and quantified with an Aperio Imagescope (Leica Biosystems, Richmond) and the necrotic area (%) was calculated using the necrosis area/total area of the liver section.

**Immunoblotting**

Cells were homogenized in a lysis buffer containing 20 mM HEPES-KOH (pH 7.9), 125 mM NaCl, 10% glycerol, 0.3% Triton™ X-100, 1 mM EDTA, 0.5% NP-40, 10 mM β-phosphoglycerate, 1 mM Na₃VO₄, 5 mM NaF, 1 mM aprotinin, 1 mM leupeptin, and 1 mM phenylmethanesulfonylfluoride. Following centrifugation, the resulting supernatants were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis after protein quantification. Samples were heated at 95 °C for 5 min and separated electrophoretically on a 12% or 14% SDS-polyacrylamide gel. Subsequently, the separated proteins were transferred to polyvinylidene fluoride membranes and incubated overnight with the indicated primary antibodies at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected with enhanced chemiluminescence lighting solution (Young in Frontier Co. Ltd, Seoul, Korea).

**Immunoprecipitation (IP) analysis**

For immunoprecipitation, HEK293 cells were lysed in a lysis buffer containing 50 mM Tris-
HCl (pH 7.5), 150 mM NaCl, aprotinin, leupeptin and 1% Nonidet P-40. The cell lysates were centrifuged, and the resulting supernatants were subjected to immunoprecipitation with antibodies against FLAG or HA, using protein G-Sepharose beads, as previously described (Woo et al., 2009). For immunoblot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE gel electrophoresis; the separated proteins were transferred to a polyvinylidene fluoride membrane, which was incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence reagents (Young in Frontier).

**Electron microscopy**

Samples were fixed for 12 h in 2% glutaraldehyde-paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.4) and washed in 0.1 M PBS. They were post-fixed with 1% OsO4 dissolved in 0.1 M PBS for 2 h, dehydrated in an ascending gradual series of ethanol and infiltrated with propylene oxide. Specimens were embedded using the Poly/Bed 812 kit (Polysciences, Warrington, PA). After embedding in pure fresh resin, the sections were polymerized at 65 °C in an electron microscopy oven (TD-700, DOSAKA, Kyoto, Japan) for 24 h. Sections of about 200~250 nm in thickness were initially cut and stained with toluidine blue (Sigma, St Louis, MO T3260) for light microscopy. Next, 70-nm thin sections were double stained with 6% uranyl acetate (EMS, Hatfield, PA, 22400) for 20 min and lead citrate (Fisher, Pittsburgh, PA) for 10 min for contrast staining. There sections were cut using a LEICA EM UC-7 (Leica Microsystems, Weltzar, Germany) with a diamond knife (Diatome) and transferred to copper and nickel grids. All thin sections were observed by transmission electron microscopy (JEM-1011, JEOL, Akishima, Japan) at the acceleration voltage of 80 kV using a Camera-Megaview III (Soft imaging system, Munster, Germany)
**Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) analysis**

Liver sections were subjected to TUNEL analysis using an *In Situ* Cell Death Detection Kit, TMR Red (Roche, Basel, Switzerland). Fluorescence signals were detected with a confocal microscope (LSM 700, Zeiss, Jena, Germany), and the frequency of apoptotic cells in the sections was quantified by determining the percentage of TUNEL-positive cells in 10 random microscopic fields per specimen.

**Antibodies and reagents**

The following antibodies were used: anti-Sirt2 (Sigma S8447); anti-p70S6K (Cell Signaling, Danvers, MS #9202); anti-phospho-p70S6K (Cell Signaling #9205); anti-S6 (Cell Signaling 2217S); anti-phospho-S6 (Cell Signaling 2211S); anti-acetylated lysine (Cell Signaling #9441); anti-Bip/Grp78 (BD Bioscience, Franklin Lakes, NJ, 610979); anti-nitrotyrosine (Millipore, Billerica, MS, 06-284); anti-mTOR (Cell Signaling #2972); anti-phospho-mTOR (Cell Signaling #2971); anti-β-actin (Abclonal, Cambridge, MA, AbC-2002-1); anti-Flag (Millipore MAB3118); anti-HA (Bethyl Laboratories, Montgomery, TX, A190-108A). APAP and dimethyl sulfoxide (DMSO), cycloheximide (CHX), chloroquine (CQ), and MG132 were purchased from Sigma Aldrich.

**Expression Vectors**

Flag-tagged human Sirt2 and the Sirt2 catalytic mutant (H187Y) cDNA were kindly provided by Dr. Ahn. HA-tagged S6K1 cDNA was purchased from Addgene (Cambridge, MA). S6K1 cDNA was purchased from the Korea Laboratory Accreditation Scheme (KOLAS).

**Cell culture**
HEK293 cells and normal liver cells (AML12 cells) were maintained under 5% CO\textsubscript{2} at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum, 1% penicillin, and 1% streptomycin.

**Quantitative RT-PCR analysis**

Total RNA was isolated from cultured cells using the TRIzol® reagent and reverse transcribed with a TAKARA cDNA synthesis kit (TAKARA, Shiga, Japan). The resulting cDNA was subjected to quantitative PCR analysis using SYBR® Green and an ABI PRISM 7700 system. Ribosomal RNA (18S) was used as an internal control (Bae et al., 2013). The sequences of the primers for mouse cDNAs (forward and reverse, respectively) were as follows: Grp78 primers, 5’-GAAAGGATGGTTAATGATGCTGAG-3’ and 5’-GTCTTCAATGTCCGATCCTCTG-3’; PERK, 5’-TCTTGGTGGTGGCTGTGAAT-3’ and 5’-GATGTTCTTGTGTAGTGGGG-3’; ATF4, 5’-ACACAGCCCTCCACCTCC-3’ and 5’-CACGGGAACCACCTGGGAG-3’; IRE1α, 5’-CTGTGGTCAAGATGGACTGGG-3’ and 5’-GAAGCGGAAACCACCTGGGAG-3’; 18S, 5’-CGCTCCAAGATCCCAACTAC-3’ and 5’-CTGAGAAACCGCTACCACATC-3’. Real-time PCR conditions were as follows: pre-denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min.

**Supplementary Figure Legends**

**Supplementary Fig.1. APAP decreases the protein levels of Sirt2**

(A) Normal liver cells (AML12 cells) treated with APAP (10 mM) and incubated with or without CHX (5 μg/ml) were lysed and subjected to immunoblot analysis with antibodies against Sirt2 and β-actin (loading control). (B-C) Densitometric analysis of Sirt2 isoform1 and 2 immunoblots obtained. (D) AML12 cells treated with APAP and incubated with or
without CQ were lysed and subjected to immunoblot analysis with antibodies against Sirt2 and β-actin (loading control). (E-F) Densitometric analysis of Sirt2 isoform1 and 2 immunoblots obtained. (G) AML12 cells treated with APAP and incubated with or without MG132 were lysed and subjected to immunoblot analysis with antibodies against Sirt2 and β-actin (loading control). (H-I) Densitometric analysis of Sirt2 isoform1 and 2 immunoblots obtained. Data are presented as means ± SD from three independent experiments. **p<0.01, *p<0.05, and N.S, not significant.

Supplementary Fig.2. The ablation of Sirt2 ameliorates the APAP-induced liver injury in the mouse liver
Increased liver injury in mouse liver with treatment of APAP for 6h is determined. (A) Representative images from H&E and Masson’s trichrome staining (magnification, ×100). (B) Quantitation of Necrosis area in H&E staining of mouse liver sections. (C) TUNEL analysis of liver sections (D) Quantitation of TUNEL analysis. (E) Serum levels of ALT (=GPT). (F) Serum levels of AST (=GOP). Data are mean ± SD from three independent experiments. *p<0.05.

Supplementary Fig.3. The ablation of Sirt2 alleviates APAP-induced liver injury and mitochondrial damage in mouse liver
Increased liver injury in mouse liver with treatment of APAP for 12h is determined. (A) 3-nitrotyrosine (3-NT) measured by immunohistochemical (IHC) stating. (B) Quantitation of IHC analysis. (C) Mitochondrial detection using electron microscopy (EM). Data are presented as means ± SD from three independent experiments. **p<0.01.

Supplementary Fig.4. The ablation of Sirt2 attenuates APAP-induced liver in mouse liver
liver

Increased liver injury in mouse liver with treatment of APAP for 6h is determined. (A) 3-nitrotyrosine (3-NT) measured by immunohistochemical (IHC) stating. (B) Quantitation of IHC analysis. Data are presented as means ± SD from three independent experiments. *p<0.05.

Supplementary Fig.5. The pharmacological inactivation of Sirt2 alleviates APAP-induced liver injury in mouse liver

Increased hepatotoxicity in mice with treatment of vehicle, APAP, or AGK2 (Sirt2 inhibitor) was investigated. (A) 3-nitrotyrosine (3-NT) measured by immunohistochemical (IHC) stating. (B) Quantitation of IHC analysis. (C) Mitochondrial detection using electron microscopy (EM). Data are presented as means ± SD from three independent experiments. **p<0.01.

Supplementary Fig.6. Ablation of Sirt2 attenuates ER stress in the APAP-induced liver injury in mouse.

(A) Immunoblot analysis of Sirt2, p-S6K1, S6K1, and β-actin (loading control) in mice with treatment of vehicle or APAP. (B) Densitometric analysis of p-S6K1/ S6K1 immunoblots was obtained. Total mRNA isolated liver from mice treated as described in (A) was subjected to qRT-PCR analysis for mRNAs of BiP/Grp78 (C) and ATF4 (D). Data are mean ± SD from three independent experiments. *P<0.05.

Supplementary Fig.7. Sirt2 has no effect in phosphorylation of mTOR

(A) Immunoblot analysis of p-mTOR and mTOR in mice with treatment of vehicle or APAP.
(B) Densitometric analysis of p-mTOR/mTOR immunoblots was obtained. (C) Immunoblot analysis of p-mTOR and mTOR in mice with treatment of vehicle, APAP, or AGK2 (Sirt2 inhibitor) (D) Densitometric analysis of p-mTOR/mTOR immunoblots was obtained. Data are mean ± SD from three independent experiments. N.S, not significant.

Supplementary Fig.8. Model for the role of Sirt2 inactivation in APAP-induced liver injury in mouse

See text for details.
Supplementary Fig. 1.
Supplementary Fig. 2.
Supplementary Fig. 3.
Supplementary Fig. 5.
Supplementary Fig. 6.
Supplementary Fig. 7.
Supplementary Fig. 8.