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Sirtuin 2 Aggravates Postischemic Liver Injury by Deacetylating Mitogen-Activated Protein Kinase Phosphatase-1

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Sirtuin 2 (Sirt2) is known to negatively regulate anoxia-reoxygenation injury in myoblasts. Because protein levels of Sirt2 are increased in ischemia-reperfusion (I/R)-injured liver tissues, we examined whether Sirt2 is protective or detrimental against hepatic I/R injury. We overexpressed Sirt2 in the liver of C57BL/6 mice using a Sirt2 adenovirus. Wild-type and Sirt2 knockout mice were subjected to a partial (70%) hepatic ischemia for 45 minutes, followed by various periods of reperfusion. In another set of experiments, wild-type mice were pretreated intraperitoneally with AGK2, a Sirt2 inhibitor. Isolated hepatocytes and Kupffer cells from wild-type and Sirt2 knockout mice were subjected to hypoxia-reoxygenation injury to determine the *in vitro* effects of Sirt2. Mice subjected to I/R injury showed typical patterns of hepatocellular damage. Prior injection with Sirt2 adenovirus aggravated liver injury, as demonstrated by increases in serum aminotransferases, prothrombin time, proinflammatory cytokines, hepatocellular necrosis and apoptosis, and neutrophil infiltration relative to control virus-injected mice. Pretreatment with AGK2 resulted in significant improvements in serum aminotransferase levels and histopathologic findings. Similarly, experiments with Sirt2 knockout mice also revealed reduced hepatocellular injury. The molecular mechanism of Sirt2's involvement in this aggravation of hepatic I/R injury includes the deacetylation and inhibition of mitogen-activated protein kinase phosphatase-1 and consequent activation of mitogen-activated protein kinases. *Conclusion:* Sirt2 is an aggravating factor during hepatic I/R injury. (HEPATOLOGY 2017;65:225-236).

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Ischemia followed by reperfusion (I/R) in the liver is a source of morbidity and mortality after liver transplantation, resection surgery, or shock and in other clinical settings.[1] At the cellular level, shortly after ischemia, adenosine triphosphate contents are rapidly depleted in hepatocytes. To survive during an ischemic period, cells switch their cellular metabolism from aerobic to anaerobic pathways, and this metabolic change causes various hepatocellular dysfunctions.[2] Upon revascularization, reoxygenation of the ischemic tissue generates various reactive oxygen species (ROS), which further contribute to profound hepatocellular

Abbreviations: Ad, adenovirus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; H/R, hypoxia-reoxygenation; IL, interleukin; I/R, ischemia-reperfusion; KO, knockout; MAPK, mitogen-activated protein kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; MPO, myeloperoxidase; mRNA, messenger RNA; ROS, reactive oxygen species; Sirt2, sirtuin 2; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.

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injury—a phenomenon known as reperfusion injury.[3] Experimental evidence suggests that Kupffer cells are responsible for ROS generation in the early phase of reperfusion injury (up to 2 hours after reperfusion).[4,5] In addition to ROS, Kupffer cells produce and secrete proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6.[6] These cytokines in turn attract and activate neutrophils during the late phase (6 hours after reperfusion) of reperfusion injury.[7] Once neutrophils are recruited into the ischemic area, they further release ROS, cytokines, myeloperoxidase (MPO), and various other mediators, all of which amplify the hepatocellular damage.[8,9] Thus, I/R is a series of events that result in cell death by apoptosis and/or necrosis and serious dysfunction in hepatocytes. Despite intensive studies, interventions with clinically proven efficacy remain to be developed.

Many of these events are regulated by signaling molecules able to sense cellular metabolic stress. Sirtuins (Sirts 1-7) are good examples of such molecules, among which Sirt1 has garnered great attention because it increases expression of antioxidant proteins and decreases apoptosis and inflammation through direct deacetylation of related proteins.[10,11] Indeed, overexpression or activation of Sirt1 protects the heart,[12] liver,[13] kidney,[14] and brain[15] against I/R injury. Conversely, reduced Sirt1 activity contributes to cell death following oxidative stress.[16] In contrast, Sirt2, another nuclear form of sirtuin, is up-regulated by anoxia-reoxygenation; and down-regulation of Sirt2 protects against anoxia-reoxygenation injury in heart-derived myoblasts.[17] Likewise, the Sirt2 inhibitor AGK2 exhibits neuroprotective effects in a cellular model of Parkinson disease.[18] However, it remains unknown whether Sirt2 modulates I/R injury in *in vivo* conditions. Drawing from these studies, we focused on the potential role of Sirt2 overexpression on oxidative stress and inflammatory responses during hepatic I/R injury in mice. We also investigated the potential benefits of pharmacologic Sirt2 inhibition and genetic deletion of Sirt2 against hepatic I/R injury. We found that Sirt2 overexpression exaggerated hepatic I/R injury by increasing neutrophil recruitment and cytokine production, whereas Sirt2 inhibition, either by genetic deletion or by pharmacological inhibition, alleviated hepatic I/R injury. More specifically, Sirt2 aggravates hepatic I/R injury by deacetylating mitogen-activated protein kinase phosphatase-1 (MKP-1).

Materials and Methods

ANIMALS

Sirt2 knockout (KO) mice (B6.129-*Sirt2*^{tm1Fwa}/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). Pathogen-free C57BL/6J male mice, 6-8 weeks old, were purchased from Orient Bio (Seoul, Korea). All animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (publication 85-23, revised 2011). The current study protocol was approved by the Institutional Animal Care and Use Committee of Chonbuk National University (approval CBU 2014-1-0221).

PREPARATION OF RECOMBINANT ADENOVIRUS

Adenovirus containing Sirt2 was purchased from Vector BioLabs (Malvern, PA). Viruses (1×10^9 plaque-forming units) were intravenously administrated to mice before I/R operations.

MODEL OF PARTIAL HEPATIC I/R INJURY

Hepatic ischemia was created by occluding the portal vein, hepatic artery, and bile duct just above

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the right branch, which provide approximately 70% of the total blood supply of the liver, as described.[19]

QUANTIFICATION OF LIVER INJURY AND CYTOKINES

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a commercial kit from Asan Pharm (Seoul, Korea). Hepatocellular function was evaluated by measuring prothrombin time, which was quantified using a portable coagulometer (CoaguChek XS; Roche Diagnostics, Mannheim, Germany). Serum levels of TNF-α, IL-6 (Enzo Life Sciences, Plymouth Meeting, PA), and IL-1β (eBioscience, San Diego, CA) were measured by enzyme-linked immunosorbent assay.

ISOLATION OF PRIMARY HEPATOCYTES AND KUPFFER CELLS

Primary hepatocytes and Kupffer cells were prepared from Sirt2 KO mice and wild-type (WT) littermates, 6-8 weeks old, by perfusion with collagenase type IV (Sigma-Aldrich, St. Louis, MO). Hepatocytes were resuspended in Medium 199 supplemented with 10% fetal bovine serum, 10 units/mL penicillin, 10 μg/mL streptomycin, and 10 nM dexamethasone mixed with 42% Percoll and centrifuged for 5 minutes at 320 g. Cells were plated at 1.5×10^6 cells/well in six-well culture dishes for western blotting or apoptosis tests. Kupffer cells were isolated through an OptiPrep gradient method and purified by selective magnetic cell sorting. Kupffer cells were plated at 5×10^5 cells/well in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics in 12-well plates.

To express exogenous proteins for immunoprecipitation, HepG2 cells obtained from the American Type Culture Collection (Manassas, VA) were transfected with *Flag-Sirt2* or *p300* using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

HYPOXIA-REOXYGENATION PROTOCOL

Primary hepatocytes, Kupffer cells, and HepG2 cells were incubated at 37°C in anaerobic jars (Oxoid, Basingstoke, UK) with oxygen-absorbing packs (AnaeroGen; Oxoid).

COCULTURE OF HEPATOCYTES AND KUPFFER CELLS

For a coculture system, Kupffer cells were cultured on an 8.0-μm Transwell membrane insert (BD Life Sciences, Franklin Lakes, NJ) and primary hepatocytes were cultured in a 24-well plate.

ANNEXIN V STAINING

Primary hepatocytes and HepG2 cells were cultured in anaerobic jars for 12 hours and 24 hours, respectively, and then reoxygenated for 6 hours. Cells were stained with annexin V according to the manufacturer's instructions (Invitrogen). The percentages of apoptotic cells were determined by flow cytometry performed on an Accuri flow cytometer (BD Biosciences, San Jose, CA).

WESTERN BLOT ANALYSIS

Liver homogenates or cell lysates containing 10 μg of whole-cell lysate were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk, blots were probed with primary antibodies to Sirt1, Sirt5 (Abcam, Cambridge Science Park, Cambridge, UK), Sirt2, β-actin, MKP-1 (Santa Cruz Biotechnology, Dallas, TX), Sirt4, Sirt7 (Biovision, Milpitas, CA), Sirt3, Sirt6, Bax, Bcl-2, cleaved caspase 3, c-Jun N-terminal kinase, p38, extracellular signal-regulated kinase, phosphorylated c-Jun N-terminal kinase, phosphorylated p38, Ac-lysine (Cell Signaling Technology, Beverly, MA), phosphorylated extracellular signal-regulated kinase, and glyceraldehyde 3-phosphate dehydrogenase (Bioworld Technology, St. Louis Park, MN).

HISTOLOGICAL STUDIES

Fixed liver tissues were embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin for light microscopy. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed with commercial kits (Promega, Madison, WI). Five to six random sections were investigated per slide to measure the necrotic area and determine the percentage of apoptotic cells. To measure necrotic areas, sections were

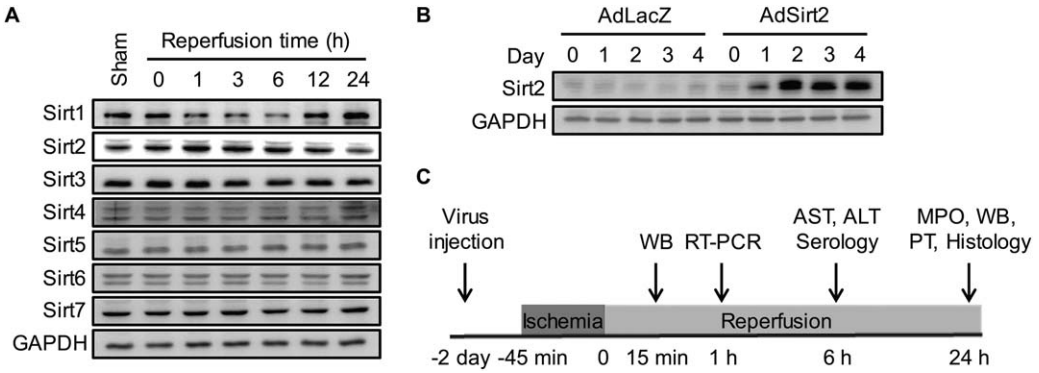

FIG. 1. Sirtuin expression during hepatic I/R injury. (A) Protein levels of sirtuins in reperfused liver tissues were analyzed by western blotting. (B) After virus injection, liver tissue homogenates prepared at the indicated time points were subjected to western blotting. (C) Blood and liver tissues were collected, and the following analyses were performed: western blotting for MAPKs (15 minutes); real-time reverse-transcription polymerase chain reaction for proinflammatory mediators (1 hour); AST, ALT, and enzyme-linked immunosorbent assay for cytokines (6 hours); MPO and western blotting for apoptotic proteins, prothrombin time, and end-point histology (24 hours). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PT, prothrombin time; RT-PCR, reverse-transcription polymerase chain reaction; WB, western blotting.

observed under an Axiovert 40 CFL microscope (Carl Zeiss, Oberkochen, Germany) and measured using iSolution DT 36 software (Carl Zeiss). For immunofluorescence, sections were immunostained with antibody against F4/80 (Abcam).

LIVER NEUTROPHIL ACCUMULATION

A naphthol AS-D chloroacetate esterase kit (Sigma-Aldrich) was used for neutrophil esterase staining of liver sections.[20] Liver MPO activity was analyzed as a measure of neutrophil accumulation.

RNA ISOLATION AND REAL-TIME REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION

Total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen). First-strand complementary DNA was generated using the random hexamer primer provided in the first-strand complementary DNA synthesis kit (Applied Biosystems, Foster City, CA). Specific primers for each gene (Supporting Table S1) were designed using Primer Express software (Applied Biosystems).

STATISTICAL ANALYSIS

Data are expressed as means ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance and Duncan's test through GraphPad Prism, version 5.02. Differences with a $P < 0.05$ were considered statistically significant.

ADDITIONAL METHODS

Detailed methods are provided in the Supporting Information.

Results

SIRT2 EXPRESSION IS INCREASED DURING HEPATIC I/R INJURY

To determine whether sirtuins play a role in hepatic I/R injury, we first determined the protein levels of all sirtuins (Sirts 1-7) in reperfused liver tissues after 45 minutes of ischemia with various lengths of reperfusion (Fig. 1A). Upon reperfusion, Sirt1 began to decrease until 6 hours and then returned to normal levels, whereas Sirt2 increased from 1 hour after the initiation of reperfusion up to 3 hours and then declined thereafter. Other sirtuins were unaffected by I/R injury. As Sirt2 was increased by I/R injury, we focused on Sirt2 in subsequent experiments. To assess the role of Sirt2 in liver I/R injury, we injected C57BL/6 mice intravenously with 1×10^9 plaque-forming units of adenovirus (Ad) LacZ or AdSirt2 virus. Liver tissues were collected at various time

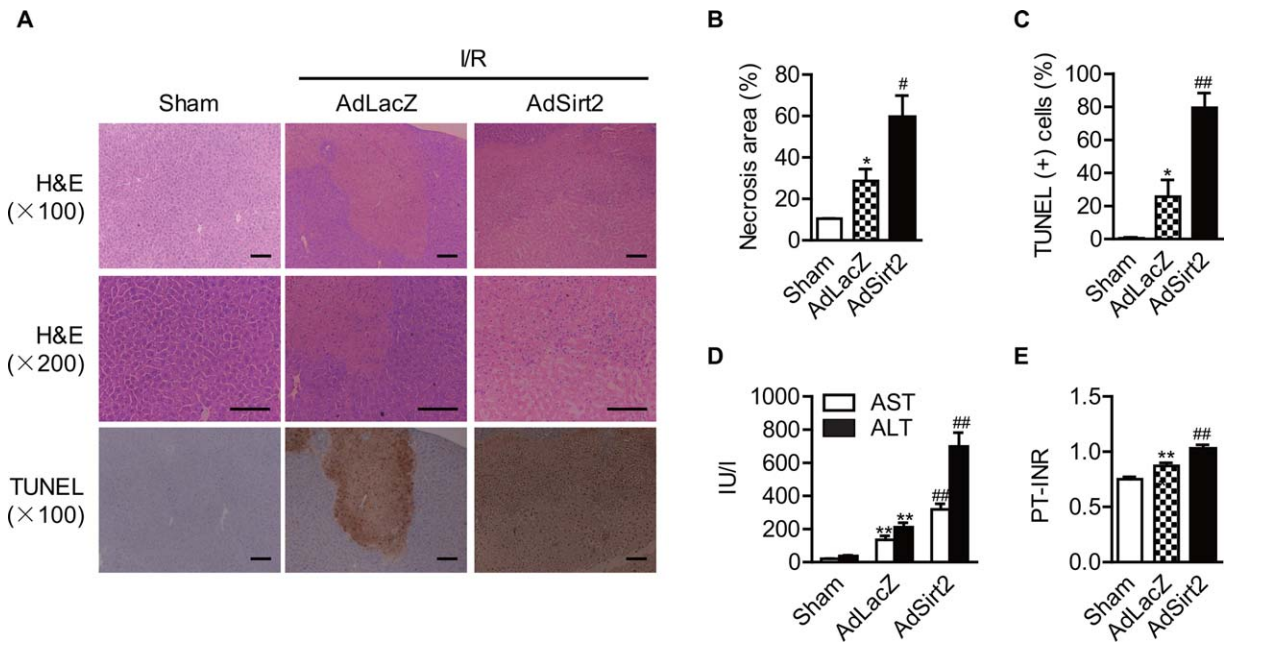

FIG. 2. Aggravation of hepatic I/R injury by Sirt2 overexpression. (A) After 24 hours of reperfusion, liver necrosis and apoptosis were assessed by hematoxylin and eosin and TUNEL staining, respectively. Bars = 100 μm. (B) The area of necrosis was measured. (C) Apoptotic cells were counted and expressed as a percentage of total hepatocytes. (D) After 6 hours of reperfusion, serum levels of AST and ALT were analyzed. (E) After 24 hours of reperfusion, prothrombin time was measured. Values are expressed as mean ± standard error of the mean (n = 4-7 mice per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham-operated mice; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus AdLacZ-injected mice. Abbreviations: H&E, hematoxylin and eosin; PT-INR, prothrombin time international normalized ratio.

points after Ad injection to analyze Sirt2 expression. Western blotting analysis showed that Sirt2 expression peaked 2 days after virus injection and then maintained its level over the next few days (Fig. 1B). To determine the cellular localization of Sirt2 expression in the liver, we performed immunohistochemistry experiments. Mice injected with AdSirt2 showed immunoreactivity for Sirt2 in hepatocytes and Kupffer cells (Supporting Fig. S1). On the basis of the production patterns of Ad-mediated Sirt2 in the liver, I/R was performed 2 days after Ad administration (Fig. 1C). Virus injection itself did not increase aminotransferase or proinflammatory cytokine levels (data not shown).

SIRT2 OVEREXPRESSION AUGMENTS HEPATIC I/R INJURY

The effects of Sirt2 overexpression on partial hepatic I/R injury were investigated. Liver injury was assessed by histologic observation. Mice subjected to sham surgery had normal liver architecture. In AdLacZ-injected mice, hepatocellular necrosis and sinusoidal congestion were observed after 45 minutes of ischemia and 24 hours of reperfusion (Fig. 2A,B). However, these mice retained considerable areas of normal liver architecture. Obviously, more extensive hepatocellular necrosis was observed in AdSirt2-injected mice than in AdLacZ-injected mice.

Although the major cause of cell death during hepatic I/R injury is necrosis, apoptotic cell death is also observed during the reperfusion process.[21] The number of apoptotic cells in I/R-injured liver tissues was determined by TUNEL staining. The number of TUNEL-positive apoptotic cells was markedly increased in AdSirt2-injected mice relative to that of AdLacZ-injected mice (Fig. 2A,C). Consistently, increased protein levels of proapoptotic Bax and cleaved caspase-3 and decreased protein levels of antiapoptotic Bcl-2 were observed in Sirt2-overexpressing mice (Supporting Fig. S2).

Biochemical parameters following I/R were consistent with histological findings of hepatic injury. Serum levels of AST and ALT were significantly increased after I/R

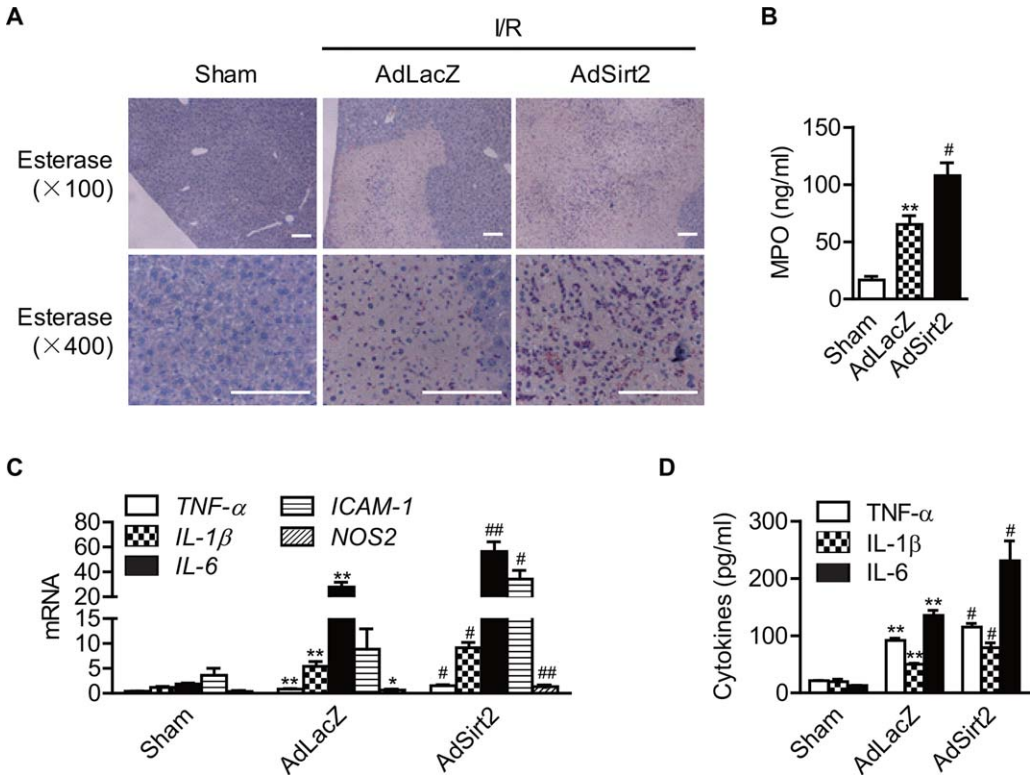

FIG. 3. Increases in neutrophil infiltration and proinflammatory mediator production in response to Sirt2 overexpression. (A) Naphthol AS-D chloroacetate esterase staining was performed on liver sections after 24 hours of reperfusion. Neutrophils are colored purple. Bars = 100 μm. (B) MPO activity was measured, as an index of neutrophil infiltration. (C) After 1 hour of reperfusion, mRNA levels of proinflammatory mediators in liver tissues were analyzed by real-time reverse-transcription polymerase chain reaction. (D) After 6 hours of reperfusion, serum levels of TNF-α, IL-1β, and IL-6 were measured using enzyme-linked immunosorbent assay. Values are expressed as mean ± standard error of the mean (n = 6-13 mice per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham-operated mice; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus AdLacZ-injected mice. Abbreviations: ICAM-1, intercellular cell adhesion molecule 1; NOS2, nitric oxide synthase 2.

injury in AdLacZ-injected mice compared to those of sham mice (Fig. 2D). Overexpression of Sirt2 in the liver greatly augmented the elevation of serum AST and ALT levels. In addition, prothrombin time was measured as a marker of liver synthetic function. Consistently, it was significantly prolonged in AdSirt2-injected mice compared to AdLacZ-injected mice (Fig. 2E).

SIRT2 OVEREXPRESSION INCREASES NEUTROPHIL INFILTRATION IN I/R-INJURED LIVER TISSUE

Accumulation of activated neutrophils in the liver plays an important role in hepatocyte death during reperfusion.[22] We used naphthol AS-D chloroacetate esterase staining, which is specific for cells of granulocytic lineage, to detect neutrophil infiltration. After 24 hours of reperfusion, the number of infiltrating neutrophils was increased in the AdLacZ-injected mice, whereas the sham mice showed little neutrophil accumulation (Fig. 3A). Consistent with an increase in liver damage, Sirt2-overexpressing mice had massive neutrophil accumulation after 24 hours of reperfusion. The MPO assay, which measures MPO, an enzyme predominantly stored in azurophilic neutrophil granules, was used to quantify neutrophil infiltration in the liver. AdSirt2-injected mice had almost twice as much MPO activity as did AdLacZ-injected mice (Fig. 3B).

Proinflammatory cytokines up-regulate adhesion molecules and cause neutrophils to infiltrate reperfused liver tissue.[1] To further examine the role of cytokines in hepatic I/R injury, messenger RNA (mRNA) levels

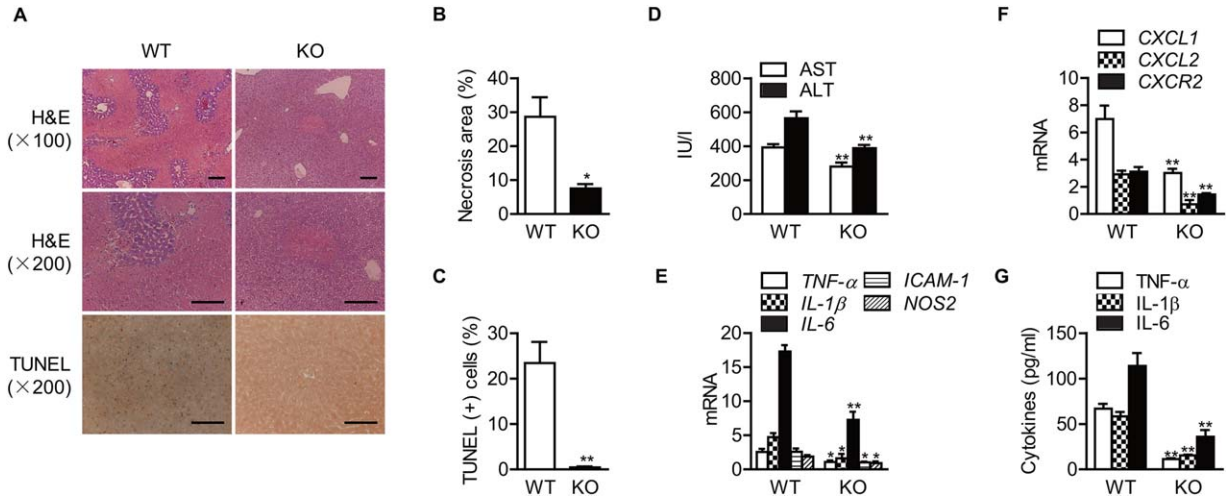

FIG. 4. Attenuation of hepatic I/R injury in Sirt2 KO mice. (A-C) WT and Sirt2 KO mice were subjected to 45 minutes of ischemia and 24 hours of reperfusion. Liver necrosis and apoptosis were assessed by hematoxylin and eosin and TUNEL staining, respectively. Bars = 100 μm. The area of necrosis was measured, and TUNEL-positive apoptotic cells were counted and expressed as a percentage of total hepatocytes. (D) After 6 hours of reperfusion, serum aminotransferase levels were analyzed. (E-G) Liver and serum levels of proinflammatory mediators were analyzed as described in the legend of Fig. 3. Values are expressed as mean ± standard error of the mean (n = 4 mice per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus WT. Abbreviations: CXCL1/2, chemokine (C-X-C motif) ligands 1 and 2; CXCR2, cysteine-X-cysteine receptor 2; H&E, hematoxylin and eosin; ICAM-1, intercellular cell adhesion molecule 1; NOS2, nitric oxide synthase 2.

of TNF-α, IL-1β, IL-6, intercellular cell adhesion molecule 1, and nitric oxide synthase 2 were measured by real-time reverse-transcription polymerase chain reaction. We observed a significant increase in mRNA levels of the aforementioned cytokines after hepatic I/R injury (Fig. 3C). Prior injection with AdSirt2 resulted in greater increases of these cytokines. In addition, changes in serum TNF-α, IL-1β, and IL-6 levels were similar to those seen in mRNA expression (Fig. 3D).

SIRT2 SUPPRESSION ATTENUATES HEPATIC I/R INJURY

Because Sirt2 overexpression aggravates hepatic I/R injury, we pretreated mice with AGK2, a Sirt2-specific inhibitor, before I/R injury. In mice that were pretreated with AGK2, the area of necrosis was significantly lower than that in the vehicle group (Supporting Fig. S3A,B). Serum levels of aminotransferases corroborated the histological findings (Supporting Fig. S3C).

When the experiments were repeated with Sirt2 KO mice, the overall results were consistent with those of AGK2 treatment. Necrotic and apoptotic areas were significantly reduced (Fig. 4A-C), and lower AST and ALT levels were detected in Sirt2 KO mice (Fig. 4D). Moreover, mRNA and the secretion of cytokine/chemokine levels were also significantly down-regulated in Sirt2 KO mice (Fig. 4E-G).

SIRT2 DEACETYLATES MKP-1 AND ACTIVATES MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING PATHWAYS

Previous studies reported that mitogen-activated protein kinase (MAPK) pathways (p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase) are activated upon hepatic reperfusion.[23,24] To determine whether Sirt2 exerted a hepatotoxic effect through activation of MAPKs, we measured the phosphorylation of three MAPKs. I/R injury resulted in increases in phosphorylation of all three MAPKs, and Sirt2 overexpression further increased these phosphorylations (Fig. 5A). We wanted to determine whether the changes in the phosphorylation of MAPKs induced by Sirt2 were mediated by suppression of the relevant phosphatase,

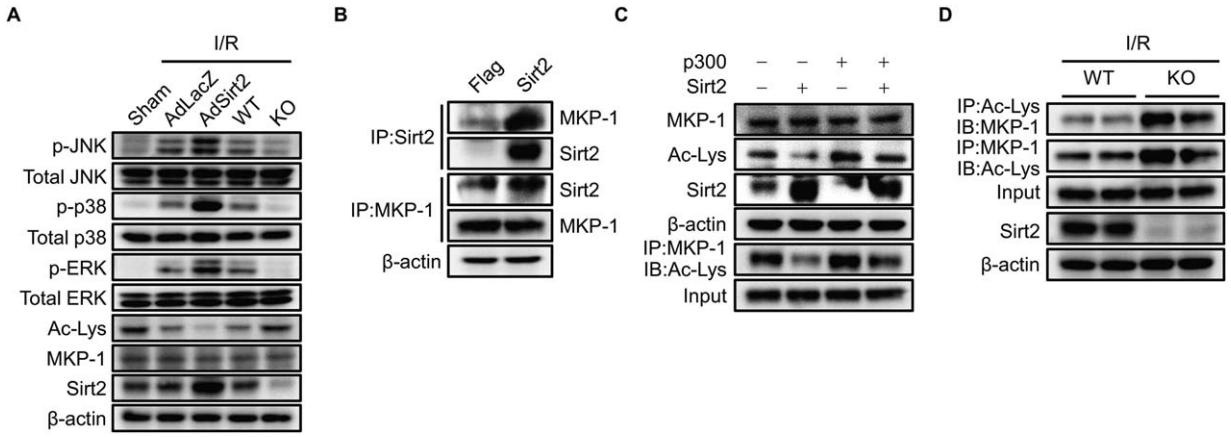

FIG. 5. Suppression of I/R-induced MKP-1 acetylation by Sirt2 overexpression. (A) After 15 minutes of reperfusion, proteins were extracted from liver homogenates. Western blotting for MAPKs, phosphorylated MAPKs, MKP-1, and acetylated lysine was performed. (B) HepG2 cells were transfected with *Flag* or *Sirt2*, and the interaction between Sirt2 and MKP-1 was analyzed. (C) HepG2 cells were transfected with *p300* or *Sirt2*, and expression and acetylation of MKP-1 were analyzed. (D) Protein extracted from livers of WT and Sirt2 KO mice was used for immunoprecipitation and immunoblotting. Abbreviations: ERK, extracellular signal-regulated kinase; IB, immunoblotting; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; p-, phosphorylated.

i.e., MKP-1. Regardless of I/R injury or genetic manipulation of Sirt2, the expression levels of MKP-1 were not altered (Fig. 5A). Because acetylation of MKP-1 enhances its interaction with p38 and interrupts MAPK signaling,[25] we next measured the acetylation of MKP-1 in I/R-injured liver tissues. MKP-1 is acetylated in sham mice; however, I/R triggered deacetylation of MKP-1 (Fig. 5A). Consistent with the findings of Jung et al.,[26] Sirt2 overexpression potentiated deacetylation of MKP-1 after I/R injury, whereas Sirt2 deletion restored acetylation of MKP-1 to the level of sham mice (Fig. 5A). To further investigate the role of Sirt2 in MKP-1 acetylation, we conducted *in vitro* overexpression studies. The results of coimmunoprecipitation assays demonstrated that Sirt2 directly binds to MKP-1 under basal conditions and that the binding was further augmented by Sirt2 overexpression in HepG2 cells (Fig. 5B). Moreover, Sirt2 overexpression in cells decreased the acetylation of MKP-1, while p300 overexpression abolished this effect (Fig. 5C). In agreement with this finding, MKP-1 acetylation was increased in Sirt2 KO liver tissues after I/R injury (Fig. 5D), implying that MKP-1 is a substrate for Sirt2-mediated deacetylation. To obtain the functional consequence of MAPK activation in Sirt2-overexpressed mouse liver, we pretreated mice with specific MAPK inhibitors and then exposed them to I/R injury. Pretreatment with specific MAPK inhibitors almost completely prevented the aggravating effects of Sirt2 overexpression on I/R-induced hepatocellular damage in mice (Supporting Fig. S4A-C).

SIRT2 SUPPRESSION DECREASES APOPTOSIS AFTER HYPOXIA-REOXYGENATION INJURY IN PRIMARY HEPATOCYTES

To further validate the attenuating activity of Sirt2 suppression in I/R-injured liver tissue, we performed *in vitro* studies using a hypoxia-reoxygenation (H/R) model. Primary hepatocytes isolated from WT mice were cultured in anaerobic jars for 3 hours and reoxygenated for various time periods. They were harvested at each time point, and their Sirt2 expression levels were compared. Similar to the results observed in I/R-injured liver tissues, H/R increased protein levels of Sirt2 (Fig. 6A). In addition, H/R markedly increased apoptosis as evidenced by increases in annexin V-positive cell numbers and in proapoptotic cleaved caspase-3 and Bax and a decrease in antiapoptotic Bcl-2 (Fig. 6B,C). However, genetic deletion or pharmacological suppression of Sirt2 resulted in decreased apoptosis of hepatocytes. Once again, Sirt2 suppression attenuated the effects

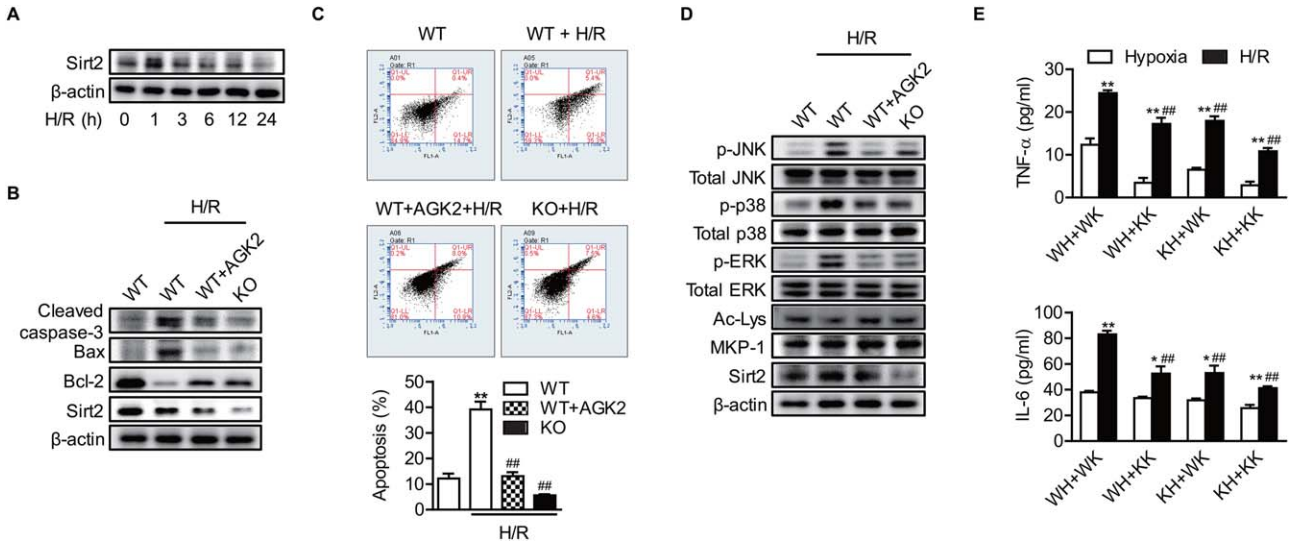

FIG. 6. Attenuation of H/R injury in hepatocytes and Kupffer cells by genetic deletion or pharmacological suppression of Sirt2. (A) Primary hepatocytes (1.5×10^6 cells/well) were isolated from C57BL/6J mice. Cells were cultured in anaerobic jars for 3 hours and reoxygenated for the indicated time periods. Protein levels of Sirt2 were determined by western blotting. (B,C) Primary hepatocytes were treated with or without 10 μM AGK2 for 2 hours. Cells were cultured in anaerobic jars for 12 hours and reoxygenated for 6 hours. Apoptosis was determined by western blotting or annexin V staining. Values are expressed as mean ± standard error of the mean (n = 3 per group). $^{**}P < 0.01$ versus WT; $^{\#\#}P < 0.01$ versus WT H/R. (D) Primary hepatocytes were cultured in anaerobic jars for 3 hours and reoxygenated for 15 minutes, and western blotting was performed. (E) Kupffer cells (1.5×10^5) were transferred to the Transwell insert and exposed to either 5-hour hypoxia or 5-hour hypoxia and 8-hour reoxygenation (H/R) in the presence of primary hepatocytes (1.5×10^5) in the lower well. Cytokines in the coculture supernatant were analyzed by enzyme-linked immunosorbent assay. Values are expressed as mean ± standard error of the mean (n = 4-6 per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus hypoxia; $^{\#\#}P < 0.01$ versus WH+WK H/R. Abbreviations: ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; KH, KO hepatocytes; KK, KO Kupffer cells; p-, phosphorylated; WH, WT hepatocytes; WK, WT Kupffer cells.

of H/R on MKP-1 acetylation and consequent activation of MAPKs (Fig. 6D). Additionally, consistent with the *in vivo* results shown in Supporting Fig. S4A-C, pretreatment with MAPK inhibitors attenuated Sirt2-mediated apoptotic cell damage in HepG2 cells (Supporting Fig. S4D,E).

I/R injury stimulates cytokine production in both hepatocytes and Kupffer cells.[6,27] We therefore isolated the respective cells from WT and Sirt2 KO mice and then exposed them to 5-hour hypoxia and 8-hour reoxygenation. Compared with WT, KO cells released significantly lower amounts of TNF-α and IL-6 in response to H/R stimulus in both hepatocytes and Kupffer cells (Supporting Fig. S5). In addition, to investigate the potential crosstalk between hepatocytes and Kupffer cells under H/R, we introduced coculture of hepatocytes and Kupffer cells isolated from both genotypes. H/R induced about 2-fold increases in the releases of TNF-α and IL-6 in coculture of WT Kupffer cells and WT hepatocytes, which is a similar degree of change relative to the culture of the respective cell types (Fig. 6E). However, when either Sirt2 KO hepatocytes and WT Kupffer cells or WT hepatocytes and Sirt2 KO Kupffer cells were cocultured, cytokine release was significantly decreased. Lastly, coculture of KO hepatocytes and KO Kupffer cells further decreased H/R-induced cytokine release.

Discussion

Growing evidence from recent studies indicates that sirtuins act in a signaling pathway that confers protection against I/R injury. For example, Sirt1 attenuates oxidative stress in the heart in response to I/R by upregulating the expression of antioxidants such as manganese superoxide dismutase and thioredoxin.[12] Ischemic preconditioning increases hepatic Sirt1 protein levels, and Sirt1 inhibition reverses the benefits of ischemic preconditioning against hepatic I/R injury.[13] Fasting for 1 day protects from hepatic I/R injury through Sirt1-dependent down-regulation of

circulating high-mobility group box 1 protein.[28] The hearts of Sirt3$^{+/-}$ mice also show less functional recovery and greater infarct size following myocardial I/R injury,[29] whereas heart-specific Sirt3 overexpression leads to increased levels of manganese superoxide dismutase and catalase.[30] Here, using gain-of-function and loss-of-function studies in mice, we have elucidated the role of Sirt2 during hepatic I/R injury and its underlying mechanisms. Specifically, our study reveals that (1) the expression of sirtuins is differentially regulated during hepatic I/R injury, Sirt2 being up-regulated and Sirt1 down-regulated; (2) Ad-mediated overexpression of Sirt2 results in greater tissue injury following I/R, whereas pharmacological inhibition or genetic deletion of Sirt2 decreases hepatic I/R injury; (3) Sirt2 deacetylates and inactivates MKP-1, which results in an increase of phosphorylation of MAPKs; and (4) Sirt2 increases apoptosis and cytokine production. These results suggest that, in contrast to Sirt1 and Sirt3, Sirt2 aggravates hepatic I/R injury.

Because apoptosis and necrosis of hepatocytes are hallmarks of I/R-induced liver injury, it is conceivable that Sirt2 may aggravate I/R injury through activation of apoptotic and necrotic pathways. In accordance with this hypothesis, our results showed enhanced apoptosis and necrosis in Sirt2-overexpressing liver tissues after I/R injury. The increases in these types of cell death seem to be associated with an increased production of proapoptotic TNF-α because we observed an increase in TNF-α in Sirt2-overexpressing liver tissue and a decrease in TNF-α in AGK2-treated liver tissue after I/R injury. Similar to these results, a recent study by Narayan et al.[31] also showed an obligate role of Sirt2 in programmed and regulated necrosis (necroptosis) in I/R-injured myocardial tissue. Complementarily, both studies suggest that Sirt2 is an important regulator of apoptosis and/or necrosis during I/R injury.

Reperfusion is followed by rapid cellular infiltration of neutrophils, which orchestrate inflammation and apoptosis.[7,8] A number of studies have reported increased expression of proinflammatory cytokines, such as TNF-α, IL-1β, and interferon-gamma, after reperfusion.[6,32,33] For this reason, strategies that interfere with neutrophil infiltration or cytokine production attenuate and even prevent reperfusion injury.[34-36] Sirt2 overexpression induced a marked infiltration of neutrophils into injured liver tissues, which might account for the proinflammatory cytokine production and apoptotic death of hepatocytes. This result is consistent with the findings of Jung et al.,[26] in which genetic deletion of Sirt2 down-regulates the expression of chemokine (C-X-C motif) ligand 2 and chemokine (C-C motif) ligand 2 and thereby suppresses neutrophil infiltration into tissues. Given the crucial role of MKP-1 in the control of expression of these cytokines and subsequent tissue inflammation,[26] the Sirt2-mediated transcriptional or post-translational changes in MKP-1 might be involved in hepatic I/R injury. Our results show no changes in MKP-1 expression levels after either Sirt2 overexpression or Sirt2 deletion. Instead, MKP-1 was deacetylated in Sirt2-overexpressing liver tissue, whereas its acetylation status was restored in Sirt2 KO liver tissue, suggesting a posttranslational regulation of MKP-1 activity by Sirt2.

MKP-1 is a dual-specificity protein phosphatase responsible for dephosphorylation of threonine and tyrosine residues that inactivates all three MAPKs.[37] Cardiac MKP-1 transgenic mice are partially protected from I/R injury, whereas MKP-1 KO mice are more sensitive to I/R injury.[38] Similarly, expression levels of MKP-1 are increased in I/R-injured human liver tissues and H_2O_2-treated HepG2 cells,[39] suggesting that genetic up-regulation of MKP-1 is an important mechanism for protecting tissues from I/R injury. In addition to transcriptional regulation, posttranslational modification of MKP-1 plays an important role in regulating its activity. Among various forms of posttranslational modification, acetylation of MKP-1 increases its interaction with substrates and decreases phosphorylation of MAPKs.[25] Our *in vitro* and *in vivo* studies clearly showed that MKP-1 is a deacetylation target of Sirt2 and that deacetylation of MKP-1 decreases its phosphatase activity. The latter finding is consistent with a study showing that deacetylation of MKP-1 increases MAPK signaling and production of inflammatory mediators in lipopolysaccharide-treated RAW264.7 cells.[25] However, there have been no studies on posttranslational modification of MKP-1 after genetic or pharmacological modulation of Sirt2 in an I/R setting. In this study, we confirmed MKP-1's involvement during reperfusion and demonstrated that its regulation includes a specific posttranslational modification.

There are some limitations of this work. First, in contrast to the increased expression of Sirt2, we observed decreased protein levels of Sirt1 after I/R injury. As described above, protective effects of Sirt1 against I/R injury have been reported; and thus, the observed hepatic I/R injury might be the result of the combined effects of Sirt1 down-regulation and Sirt2 up-regulation. The differential roles of the Sirt1 and

Sirt2 pathways remain to be elucidated. Second, Sirt2 is a member of the oxidized nicotinamide adenine dinucleotide-dependent histone deacetylases, and it can affect the expression of several genes by altering histone-regulated gene accessibility. Moreover, Sirt2 is known to deacetylate several nonhistone proteins as well. For example, Sirt2 deacetylates receptor-interacting protein 1 in response to TNF-α stimulation and triggers necroptosis.[31] It also deacetylates the p65 subunit of nuclear factor kappa B at K310 and impairs nuclear factor kappa B-mediated survival pathways.[40] Therefore, Sirt2 might worsen hepatic I/R injury by targeting other pathways in addition to MKP-1. Third, some I/R experiments were conducted with a relatively small number of mice due to the limited availability of mice; and thus, they await confirmation by further experimentation.

In summary, Sirt2 deacetylates MKP-1 and up-regulates MAPK pathways during I/R, thereby augmenting inflammatory responses and enhancing cell death. Pharmacologic and genetic suppression of Sirt2 provides additional evidence supporting this model.

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