Nicotinamide Mononucleotide, an NAD\(^+\) Precursor, Rescues Age-Associated Susceptibility to AKI in a Sirtuin 1–Dependent Manner

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ABSTRACT

The rapid growth of an aging population creates challenges regarding age-related diseases, including AKI, for which both the prevalence and death rate increase with age. The molecular mechanism by which the aged kidney becomes more susceptible to acute injury has not been completely elucidated. In this study, we found that, compared with the kidneys of 3-month-old mice, the kidneys of 20-month-old mice expressed reduced levels of the renal protective molecule sirtuin 1 (SIRT1) and its cofactor NAD\(^+\). Supplementation with nicotinamide mononucleotide (NMN), an NAD\(^+\) precursor, restored renal SIRT1 activity and NAD\(^+\) content in 20-month-old mice and further increased both in 3-month-old mice. Moreover, supplementation with NMN significantly protected mice in both age groups from cisplatin-induced AKI. SIRT1 deficiency blunted the protective effect of NMN, and microarray data revealed that c-Jun N-terminal kinase (JNK) signaling activation associated with renal injury in SIRT1 heterozygotes. In vitro, SIRT1 attenuated the stress response by modulating the JNK signaling pathway, probably via the deacetylation of a JNK phosphatase, DUSP16. Taken together, our findings reveal SIRT1 as a crucial mediator in the renal aging process. Furthermore, manipulation of SIRT1 activity by NMN seems to be a potential pharmaceutical intervention for AKI that could contribute to the precise treatment of aged patients with AKI.


It is well documented that the incidence of AKI increases with age.\(^1,2\) Multiple studies have shown that aging is an independent risk factor for AKI and its associated mortality and morbidity.\(^1–4\) Although aging-associated comorbidities and iatrogenic factors are responsible for these outcomes, age-related cellular deterioration and an impaired response of the renal cells to stress have also been recognized to increase the susceptibility of the kidney to injury.\(^5–8\)

Recent studies have shown that sirtuin 1 (SIRT1) plays an important role in the cellular response to stress and has been shown to extend the lifespan, at least in lower animals.\(^9–11\) SIRT1, an NAD\(^+\)-dependent deacetylase, is a member of the Sirtuin family, which has seven family members. SIRT1 is primarily localized to the nucleus, where it deacetylates many nuclear proteins and transcription factors. SIRT1 has been shown to underlie calorie restriction–related health benefits, including prolonged lifespan and reduced age-related deterioration.\(^12–14\) Consistently, activators of SIRT1, including resveratrol and synthetic SIRT1–activating compounds, have been shown to promote health in experimental animals,\(^15–18\) although the results from human studies are somewhat variable, probably because of the complexities of the mechanisms of their action.\(^19–22\)

NAD\(^+\) is known to mediate hydrogen transfer in the intracellular metabolic pathway in mitochondria and the redox reaction. NAD\(^+\) has also been
found to be an indispensable cofactor for several important enzymatic reactions, including SIRT1, supporting an important role of SIRT1 in modulating the cellular response to metabolism and oxidative stress. Accumulation studies suggest that cellular NAD⁺ levels reduce with aging and are accompanied by reduced SIRT1 activity. Boosting NAD⁺ is associated with lifespan extension, and restoring NAD⁺ with NAD⁺ precursors, such as nicotinamide mononucleotide (NMN) and nicotinamide riboside, corrects many metabolic abnormalities.

In this study, we examined the role of NAD⁺/SIRT1 in the susceptibility to AKI in aged animals. Our study shows that NAD⁺ and SIRT1 deficits in aged mice kidneys contributed to increased vulnerability to cisplatin-induced AKI; NMN treatment rescued the aged kidneys from cisplatin-induced AKI in an SIRT1-dependent manner. The mechanism by which NAD⁺/SIRT1 protects the kidney involves epigenetic regulation of the c-Jun N-terminal kinase (JNK) pathway. This study sheds light on the mechanisms underlying age-associated susceptibility to AKI and identifies endogenous NAD⁺ as a potential therapeutic target for AKI, particularly in the elderly.

RESULTS

Aged Kidneys Are Susceptible to Cisplatin-Induced AKI

A dose of 20 mg/kg body wt cisplatin successfully induced AKI in 3- and 20-month-old 129 mice. Kidney function was evaluated by BUN and serum creatinine (72 hours of cisplatin). BUN and creatinine levels of 20-month-old AKI mice were twice those of 3-month-old mice (Figure 1, A and B). Consistently, the histology study of the kidneys revealed a significantly worse tubular injury in the 20-month-old mice than in the 3-month-old mice, including severe dilation of the proximal tubules, cast formation, and massive detachment and necrosis of the tubular epithelium (Figure 1D). The pathological analysis assessed by the percentage of damaged tubules at 72 hours of cisplatin in a blind manner further confirmed that aged kidneys are susceptible to toxin insult (Figure 1C). Additionally, electron micrograph showed that cisplatin induced mild swelling of the mitochondria in the proximal tubules in 3-month-old mice, whereas in 20-month-old mice, the same dosage of cisplatin induced mitochondrial fragmentation into spheres and partial ridge breakdown (Figure 1E).

Aging Is Associated with Declined SIRT1 Expression and NAD⁺ Levels in the Kidney

In the kidney cortex tissue, mRNA and protein expression of SIRT1 and its substrate NAD⁺ reduced with aging (Figure 2, A–C, Supplemental Figure 1). SIRT1 expression and the NAD⁺ content in 20-month-old kidneys were approximately one third of those in the 3-month-old kidneys. We then examined the expression of the enzymes of NAD⁺ biosynthesis: nicotinamide phosphoribosyltransferase (NAMPT), nicotinamide mononucleotide adenyllytransferase1 (NMNAT1), and NMNAT3. Real-time PCR showed that the mRNA levels of all of these enzymes were significantly lower in the 20-month-old kidney cortex compared with the 3-month-old kidney cortex (Figure 2D).

NMN Supplementation Protects the Kidney from Aging-Associated Susceptibility to AKI

NMN is an intermediate product in the NAD⁺ salvage pathway. Four days of NMN supplementation restored NAD⁺ levels in both the young and aged kidney cortices (Figure 3A) and further increased SIRT1 activity as indicated by direct enzymatic activity measurement and reduced acetylation level of Foxo1, a downstream target molecule of SIRT1 (Figure 3, B and C). To explore whether NMN could rescue the mice from aging-associated susceptibility to AKI, we provided both 3- and 20-month-old mice with NMN immediately after cisplatin exposure. As shown in Figure 4, the NMN supplement significantly attenuated cisplatin-induced AKI compared with the PBS control. The serum creatinine in 20-month-old mice after cisplatin exposure was reduced by 60% in NMN-treated mice compared with vehicle-treated mice. The histology revealed ameliorated tubular necrosis and cast formation in NMN-treated AKI mice (Supplemental Figure 2). Also, NMN treatment reduced cisplatin-induced apoptosis as assessed by decreased cleaved caspase-3 expression and fewer terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL)-positive cells.

SIRT1 Deficiency Aggravates Cisplatin-Induced AKI

To determine whether the lower levels of SIRT1 observed in the aged kidney could predispose to worsen kidney injury, studies were performed in SIRT1 heterozygous mice due to embryonic and perinatal lethality of SIRT1 homozygotes. A loss of one allele of the SIRT1 gene significantly aggravated renal damage compared with in wild-type littermates after cisplatin exposure, resulting in higher BUN and serum creatinine levels, more tubular injury, and more severe mitochondrial fragmentation (Figure 5, A–E). Enhanced tubular cell damage was further confirmed by Western blotting of caspase-3, real-time PCR of apoptosis-associated genes, and TUNEL staining: SIRT1 heterozygotes showed higher cleaved caspase-3 protein levels, BAX, and caspase-3 -9 mRNA expression as well as many more TUNEL labeling apoptotic cells (Figure 5, F–I).

The Effect of NMN Is Dependent on SIRT1

To determine whether the protective effect of NMN depends on SIRT1, we examined the effect of NMN on SIRT1-deficient mice and also, equivalently damaged wild-type mice treated with higher dose of cisplatin (30 mg/kg body wt) (Supplemental Figure 3). As shown in Figure 6 and Supplemental Figure 4, NMN protected the kidney from severe renal injury in wild-type mice, but this therapeutic effect was substantially attenuated when one allele of the SIRT1 gene was deleted. Consistent in vivo data also supported that NMN rescued cisplatin-induced cell death in a SIRT1-dependent manner.
Figure 1. Aging worsens acute kidney injury induced by cisplatin. Renal function evaluated by (A) BUN and (B) serum creatinine in the indicated groups at 72 hours after saline or cisplatin treatment (n=5 for saline-treated groups and n=8 for cisplatin-treated groups). (C) Tissue damage scored by the percentage of damaged tubules in cisplatin- or saline-treated 3- and 20-month-old mouse kidneys (n=6). (D) Representative hematoxylin and eosin-stained sections at both low and high magnification. (E) EM examination of renal PTCs in the indicated groups is shown and quantified. Data are expressed as mean±SEM and were analyzed using unpaired Student’s t test between two groups and ANOVA between multiple groups followed by post-tests. Scale bars, 2 μm. *P<0.05; **P<0.01.
NMN Treatment Benefits Kidney from Ischemia-Reperfusion Injury

We further verified the therapeutic effect of NMN in an additional AKI model; 22 minutes of bilateral renal ischemia was induced in wild-type mice, and NMN or PBS was administrated before the surgery and 24 hours after reperfusion. Kidney function and histology analysis were performed at 48 hours after reperfusion. As shown in Figure 7, mice treated with NMN had much lower BUN and serum creatinine levels and improved tubular damage compared with mice treated with PBS. Therefore, beneficial effects of NMN on kidney were shown in both cisplatin- and ischemia-reperfusion–induced AKI, indicating endogenous NAD+ as a potential target for AKI treatment.

The JNK Signaling Pathway Is Activated in Age-Associated AKI

A microarray study was conducted to explore the molecular mechanism involved in age-associated susceptibility to AKI. Renal cortex samples of SIRT1-intact or -deficient mice subjected to cisplatin or saline as the control were assigned in the mRNA microarray. Each group contained three biological duplicates. Extensive pathway-network analysis of the array data pointed to MAPK/JNK signaling being responsible for increased acute renal injury in the absence of one allele of SIRT1 (Supplemental Figure 5). The microarray data were validated by Western blotting of the three main signaling modules in the MAPK pathway. JNK other than p38 or extracellular signal–regulated kinase 1/2 (ERK1/2) showed a robust activation via the enhanced phosphorylation of JNK as well as c-JUN and ATF2 (Figure 8A). In addition, JNK signaling activation was observed in the age-associated injured kidney (Figure 8B).

The JNK Inhibitor Prevents Cisplatin-Induced Apoptosis in Cultured HK-2 Cells

To determine whether JNK activation was associated with SIRT1 deficiency–enhanced cell injury, we examined the effect of JNK inhibitor on cell survival and apoptosis in cells deficient in SIRT1. Consistent with SIRT1 knockout mice, knocking down SIRT1 by siRNA in cultured HK-2 cells sensitized the cells to cisplatin (Figure 9, A and B). Transfection with SIRT1 siRNA or scrambled RNA HK-2 cells showed signs of apoptosis and the phosphorylation of JNK after exposure to cisplatin. Cell viability was decreased and cleaved caspase-3 protein expression was enhanced in cisplatin-treated SIRT1 knockdown cells compared with the control. SP600125, a JNK inhibitor, blocked JNK activation and reduced apoptosis in cisplatin-treated HK-2 cells (Figure 9, A and B). Interestingly, in addition to the protective effect of the JNK inhibitor on HK-2 cells from cisplatin-induced apoptosis, we
found activation of phosphorylated JNK in SIRT1-deficient cells without cisplatin exposure.

**SIRT1 Regulates JNK Activity through the Deacetylation of Dual-Specificity Phosphatase16**

The relationship between the loss of SIRT1 and the activation of JNK was further examined using two SIRT1 siRNAs targeting different loci of the *SIRT1* gene. Transfected with either of these two siRNAs for 48 hours, SIRT1 protein expression was suppressed in HK-2 cells (Figure 9C). JNK was phosphorylated and activated as indicated by Western blotting, which revealed the phosphorylation of ATF2. Because no direct interaction between SIRT1 and JNK was detected in the immunoprecipitation experiment (data not shown), we examined the upstream phosphor-kinase and phosphatase of JNK, because JNK signaling is a phosphorylation-dependent cascade. We investigated MKK7 and MKK4, two major JNK-phosphor-kinases, both in the total and phosphorylated form of MKK7 and MKK4 and found no alteration in the deprivation of SIRT1 (Figure 9C). To explore the related phosphatase levels, we constructed a flag-tagged dual-specificity phosphatase16 (DUSP16) plasmid and delivered it into HK-2 cells with SIRT1 siRNA or scrambled RNA, and the phosphorylation and acetylation levels of flag-DUSP16 were determined by immunoprecipitation. When SIRT1 was silenced by siRNA, the phosphorylation of flag-DUSP16 was downregulated, whereas acetylation was upregulated (Figure 9D). Additionally, in HK-2 cells, SIRT1 was coprecipitated with DUSP16 and was not coprecipitated with other JNK phosphatases and phosphor-kinases (Figure 9E). The interaction between SIRT1 and DUSP16 was further confirmed by reverse IP, in which flag-DUSP16 was found to be coprecipitated with SIRT1 (Figure 9F, Supplemental Figure 6A). This finding strongly suggested that, with the lack of SIRT1, DUSP16 would remain acetylated instead of becoming phosphorylated and lose its phosphatase activity, resulting in a constant phosphorylation of JNK. Additional experiment using lysine to arginine mutant DUSP16 plasmids identified lys<sup>462</sup> and lys<sup>482</sup> as acetylated targets of DUSP16, because SIRT1 deficiency–induced JNK phosphorylation was blunted in HK-2 cells when transfected with lys<sup>462</sup> and lys<sup>482</sup> mutant DUSP16 plasmid (Supplemental Figure 6B).
DISCUSSION

Complex factors contribute to the enhanced susceptibility to AKI with age. Comorbidities, such as diabetes, hypertension, hyperlipidemia, vascular diseases, and iatrogenic factors, such as contrast, medication, and surgical procedures, all may contribute to age-associated susceptibility to AKI. Our previous studies and those of others have shown that the longevity gene SIRT1 plays a critical role in protecting the kidney from injury. This study identified the substrate of SIRT1,
NAD$^+$, as an important factor associated with increased susceptibility to AKI among the elderly. We showed that aged kidneys have decayed NAD$^+$ metabolism and reduced SIRT1, and supplementation of NMN, an NAD$^+$ precursor, restored the NAD$^+$ content and SIRT1 activity and rescued cisplatin-induced acute renal damage. SIRT1 deficiency substantially blunted the protective effect of NMN, indicating that the renal protective effect of NMN relied on SIRT1. Thus, increasing endogenous NAD$^+$ levels could become a therapeutic target for AKI, particularly in the elderly.

A reduced level of NAD$^+$ has been detected with aging in worms, skeletal muscles and livers in mice, and skin tissues in human, although the mechanism remains unclear. The salvage pathway accounts for most of the NAD$^+$ in mammals, which is catalyzed by two rate-limiting enzymes. Briefly, NAD$^+$ is converted from NMN by NMNAT after the conversion of NMN from nicotinamide by NAMPT. Thus, a deficit in the salvage pathway could contribute to the insufficient production of NAD$^+$.

Overconsumption would also cause NAD$^+$ deficiency. PARPs and CD38, two NADases, also use NAD$^+$ as a substrate, thus consuming NAD$^+$. PARP-1 knockout is associated with elevated NAD$^+$ levels. During aging, activated PARP-1 due to increased DNA damage has been suggested to contribute to reduced NAD$^+$ levels in aged organs. A recent study revealed that CD38 level and activity increased in multiple tissues during aging and played an important role in age-associated NAD$^+$ decline. In this study, the message RNA expression levels of both NAMPT and NMNAT were lower in the kidney cortex of 20-month-old mice compared with that in 3-month-old mice, indicating that the decline of the NAD$^+$...
Figure 5. The SIRT1-deficient kidney is susceptible to AKI. Renal function evaluated by (A) BUN and (B) serum creatinine in the indicated groups (n=4 for saline-treated groups and n=8 for cisplatin-treated groups). Tissue injury assessed by (C) percentage of damaged tubules and (D) mitochondrial density in cisplatin-treated wild-type and SIRT1 heterozygotes (n=6). (E) Representative hematoxylin and eosin–stained sections of SIRT1-intact (upper panel) and -deficient (lower panel) kidneys subjected to cisplatin. Right panel indicates EM of representative renal PTCs in cisplatin-treated wild-type and SIRT1 heterozygotes. Scale bars, 2 μm. Injury-induced apoptosis determined by (F) Western blotting of cleaved caspase-3, (G) relative mRNA expression of...
biosynthetic pathway with aging at least in part contributes to the age-related NAD⁺ reduction in the kidneys. NAMPT is also a major output of circadian transcription factors BMAL and CLOCK, and declined central or peripheral circadian function with aging may contribute to decreased NAMPT in the aged mice.²⁴,⁴² Mammalian NMNAT was reported to be highly expressed in proliferating cells and tissues,⁴⁵ suggesting that the NMNAT might be decreased as an adaptive response to the reduced metabolism in the aged animals. Notably, two of three isoforms of NMNATs are found in the kidney: NMNAT1, mainly expressed in the nucleus, and NMNAT3, expressed in the mitochondria. Shrinkage of the NAD⁺ pool in the nucleus and mitochondria during aging was suggested indirectly by decreased NMNAT1 and NMNAT3 expression, but the precise sub-cellular distribution and alteration of the NAD⁺ content are not clear. Given that NAD⁺ is able to travel freely inside the cell, decreased cellular NAD⁺ levels may result in reduced activity of the enzymes that use NAD⁺ as a substrate, including SIRT1. This study shows that NMN, an intermediate product of NAD⁺ biosynthesis in the salvage pathway, replenished NAD⁺ levels in the aged kidneys and benefited the kidney with cisplatin-induced AKI, suggesting that reduced NAD⁺ levels are associated with enhanced kidney injury and that NAD⁺ may be an important intervention target for kidney protection.

NAD⁺ is required for SIRT1 activity, and SIRT1 has been previously documented as a renal survival factor.³⁸ We examined whether the protective effect of NMN depended on SIRT1. This study shows that the intraperitoneal injection of NMN boosted NAD⁺ levels in both young and aged kidneys, which was also associated with increased activity of SIRT1, and protected the kidneys from both cisplatin- and ischemia-reperfusion-induced AKI, indicating that NMN rescued AKI by activating SIRT1. Importantly, the protective effect of NMN supplementation was substantially blunted in SIRT1-deficient mice, indicating that the protective effect of NMN occurs through SIRT1 activity.

The mechanism by which SIRT1 exerts its health benefits has been extensively studied. FOXO, PGC1α, NF-κB, and HIF have been reported to be targets of SIRT1.⁴⁶ To define the signaling mechanism underlying SIRT1 deficiency–associated AKI, we applied the microarray approach using SIRT1-deficient or -intact mice exposed to cisplatin or the vehicle.
The array data indicated the activation of JNK signaling. Activation of JNK was then validated through Western blotting. Because JNK phosphorylation is observed in injured tissue, to examine whether the JNK activation is caused by tissue damage, we induced similar kidney injuries among wild-type mice (30 mg/kg cisplatin) and SIRT1-deficient mice (20 mg/kg) and found more intensive JNK phosphorylation in the heterozygotes than in the wild-type mice, indicating that the JNK activation was enhanced by SIRT1 deficiency (Supplemental Figure 3). Additionally, the administration of a JNK inhibitor attenuated cisplatin-induced apoptosis in both SIRT1-intact and -deficient HK-2 cells. In the cell culture study, JNK activation was observed when SIRT1 was knocked down by siRNA in HK-2 cells. We then examined the mechanism by which SIRT1 influences JNK. Our IP experiment failed to find a direct interaction of SIRT1 and JNK. We then examined the phosphor-kinase and phosphatases of JNK and found that DUSP16 is a potential target of SIRT1. DUSP16, belonging to a family of mitogen-activated protein kinase phosphatases (MKPs), shows a specificity preference for JNK. MKPs are inducible and act rapidly as a feedback loop of the MAPK cascade in a cell type–specific manner. Furthermore, MKPs can be modified post-transcriptionally. In HK-2 cells transfected with SIRT1 siRNA, we found an enhanced acetylation and reduced phosphorylation of DUSP16. Given that SIRT1 is a deacetylase and targets a variety of proteins in addition to histones, it is plausible that acetylated DUSP16 was present in the absence of SIRT1. More importantly, interaction between SIRT1 and DUSP16 was observed in HK-2 and 293T cells by coimmunoprecipitation. It has been documented that the phosphorylated state of MKPs is associated with their stabilization and prolonged half-life; therefore, the reduction of

Figure 7. The reno-protective effect of NMN on ischemia-reperfusion–induced AKI. Renal function evaluated by (A) BUN and (B) serum creatinine of mice 48 hours after ischemia-reperfusion injury (I/R) with or without NMN administration. (C) Histologic analysis of tubular damage and (D) representative hematoxylin and eosin–stained kidney sections are shown (n=6 for the PBS-treated group and n=7 for the NMN-treated group). Data are expressed as mean±SEM and were analyzed by unpaired Student’s t test. *P<0.05; **P<0.01.
phosphorylated DUSP16 in SIRT1 deprivation may reduce their phosphatase activity and lead to the sustained activation of JNK. Two lysines (lys462 and lys482) are closely located near the phosphorylated site, serine446 of the DUSP16 protein, prompting the possibility that acetylation might compete with phosphorylation and influence protein functioning under certain circumstances.

In summary, renal aging is associated with declined NAD+ metabolism and the consequent reduced SIRT1 activity and increased susceptibility to AKI. Supplementation with NMN, an NAD+ precursor, restores NAD+ and SIRT1 levels and protects the kidney from age-related AKI in an SIRT1-dependent manner. This study provides evidence suggesting that approaches to restore endogenous NAD+ levels are a potential therapeutic target for AKI, particularly in the elderly.

**CONCISE METHODS**

**Animals**

Wild-type 129S2/Sv and C57BL/6 mice were purchased from Vital River (Beijing, China), housed in a constant temperature room of the animal facility of Fudan University Medical Animal Center with a 12-hour dark/12-hour light circle, and allowed free access to standard rodent chow and water unless specifically indicated. SIRT1<sup>−/−</sup> mice in C57BL/6 background were generated as previously described. All animal studies were approved by the Institutional Animal Care and Use Committee of Fudan University.

**Experimental Models of AKI**

Three- and 20-month-old mice in 129S2/Sv background and SIRT1 heterozygotes and wild-type littermates in C57BL/6 background were used in the cisplatin-induced AKI model by intraperitoneal injection of cis-diammineplatinum(II) dichloride (cisplatin at 20 or 30 mg/kg; P4392; Sigma-Aldrich, St. Louis, MO) or saline as the control. Note that mice in 129 background were more sensitive to cisplatin-induced injury than mice in C57 background. The kidney cortices were harvested for further study 72 hours after cisplatin exposure. To examine the effect of NMN (β-NMN; N3501; Sigma-Aldrich), 500 mg/kg NMN or an equivalent volume of PBS was given intraperitoneally for 4 consecutive days, and the mice were euthanized 4 hours after the last injection. If the mice were also subjected to cisplatin, the first dose of NMN or PBS was given immediately after the cisplatin.

Warm ischemia-reperfusion surgery was performed on 8- to 10-week-old wild-type C57BL/6 mice. Briefly, mice were kept on a homoeothermic unit and subjected to flank incisions. Bilateral renal pedicles were exposed and clamped for 22 minutes. After removal of the clamp, color of kidneys turned from dark purple to pink. The animals were allowed to recover and euthanized 48 hours after reperfusion. NMN (500 mg/kg body wt) or equivalent amount of PBS was administrated right before the procedure and 24 hours after reperfusion by intraperitoneal injection.

**Renal Function Evaluation**

Renal function was evaluated by BUN and creatinine. BUN was measured using the UREA KIT (liquid; UV-GLDH method; Shanghai
Figure 9. SIRT1 regulates JNK signaling via the deacetylation of DUSP16. HK-2 cells were transfected with scrambled RNA or SIRT1 siRNA and treated with cisplatin alone, cisplatin and SP600125, or cisplatin and NMN as indicated. (A) Western blotting analysis of SIRT1, P-JNK, and cleaved caspase-3 is shown. β-Actin protein expression was used as the loading control. Expression of cleaved caspase-3 in each experimental group was quantified by densitometry. (B) Cell viability was determined using CCK-8 assay (n=3–5). Data are expressed as mean±SEM and were analyzed using ANOVA followed by post-tests. *P<0.05; **P<0.01. (C) JNK signaling and major phosphokinase and phosphatase of JNK were examined by immunoblotting in the HK-2 cells.
Kidney samples were fixed in 4% paraformaldehyde, and paraffin-embedded sections were stained with hematoxylin and eosin. Over 20 high-power fields of each section were reviewed and scored for tubular injury in a single-blind fashion. The pathologic assessment was performed on the basis of the percentage of tubules with necrosis, detachment, cast formation, dilation, or cell swelling.

Electron Microscopy

Tissues of the kidney cortex were fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffer. After a standard embedding procedure, the proximal tubular cells were examined and photographed under a transmission electron microscope. Mitochondrial density was estimated using morphometric analysis according to Weibel.51

TUNEL Assay

Apoptotic cells were detected by TUNEL assay (G3250; Promega, Fitchburg, WI) according to the manufacturer’s instructions and quantified by numbers of TUNEL-positive cells in ten fields per section and five sections per kidney. DAPI was counterstained.

Real-Time PCR

Total RNA was extracted from the kidney tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA), and 2 μg RNA was reverse transcribed using oligo dT as primer according to the manufacturer’s instructions (K1622; Fermentas, Thermo Fisher Scientific). Real-time PCR was performed using the SYBR Green Premix Kit (RR820; Takara Bio, K1622; Fermentas, Thermo Fisher Scientific) according to the protocol as previously described.50

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NAD+ Measurement

NAD+ from the kidney cortex was measured with an NAD/NADH Quantitation Kit (K337; BioVision, Milpitas, CA) according to the manufacturer’s instructions.

Western Blotting

Proteins from frozen kidney tissue and cultured HK-2 cells were extracted using RIPA lysis buffer (P0013B; Beyotime, Nantong, China) with a 1:100 phosphatase inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany), a 1:100 protease inhibitor cocktail tablet (Roche), and 1:100 100 mM PMSF (ST506; Beyotime). Protein concentration was determined using the Bradford protein assay (P0111; Beyotime). After the separated proteins on SDS-PAGE were transferred electrophoretically to PVDF membranes (Millipore, Billerica, MA) and blocked by 5% nonfat milk or BSA, immunoblotting was performed using specific antibodies overnight at 4°C: anti-mouse SIRT1 (1:5000; Abcam, Cambridge, United Kingdom), anti–caspase-3 (1:500; CST, Danvers, MA), anti–β-actin (1:5000; CST), anti–ac-P53 (1:1000; CST), anti–ac-Foxo1 (1:1000; CST), anti–P-JNK (1:1000; CST), anti–JNK (1:1000; CST), anti–P-p38 (1:1000; CST), anti–p38 (1:1000; CST), anti–P-ERK1/2 (1:1000; CST), anti–ERK1/2 (1:1000; CST), anti–P-ATF2 (1:1000; CST), anti–P-cJUN (1:1000; CST), anti–human SIRT1 (1:4000; Abcam), anti–flag (1:1000; Sigma-Aldrich), antiphosphoserine/threonine (1:1000, Abcam), antiacycetated-lysine (1:1000; CST), anti–MKK7 (1:1000; CST), anti–P–MKK7 (1:1000; CST), anti–MKK4 (1:1000; CST), anti–P–MKK4 (1:1000; CST), anti–DUSP3 (1:5000; Abcam), and anti–DUSP6 (1:1000; Abcam). Then, the membranes were incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse Ig for 1 hour at room temperature. Antibody labeling on the Western blots was visualized using a chemiluminescence reagent (WBKLS0100; Millipore) with the GE ImageQuant LAS 4000. Densitometry analysis was performed using ImageJ software.

Quantification of SIRT1 Activity

Nuclear protein was extracted from the kidney cortex and used to quantify the activity of the total SIRT1 enzymes using commercially available kits according to the manufacturer’s instructions (ab113474 for nuclear protein extraction; Abcam and ab156915 for SIRT1 activity measurement; Abcam).

Immunoprecipitation

HK-2 cells were collected and lysed in IP buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, and 1 μg/ml leupeptin) supplemented with a 1:100 proteinase inhibitor cocktail tablet (Roche) and 1:100 100 mM PMSF (ST506; Beyotime). Then, immunoprecipitation was performed by adding antibody coated with Protein G plus/Protein A Agarose Beads (IP05; Millipore) to the protein lysate transfected with scrambled RNA or SIRT1 siRNA. (D) Flag-DUSP16 plasmid and SIRT1 siRNA or scrambled RNA were delivered together into HK-2 cells. Phosphorylation and acetylation levels of DUSP16 were determined with an immunoblotting examination of immunoprecipitated flag. The interaction of SIRT1 and DUSP16 was determined by (E) immunoprecipitation of SIRT1 in HK-2 cells and (F) reverse immunoprecipitation of flag in HK-2 cells transfected with flag-tagged DUSP16 plasmid. The data are representative of three independent experiments. 
at 4°C for 5 hours. Endogenous SIRT1 protein was immunoprecipitated using an anti-SIRT1 antibody (Abcam), flag-DUSP16 was immunoprecipitated using an antilag antibody (Sigma-Aldrich), and nonspecific anti-rabbit or -mouse Ig was used as the control. Western blotting was conducted to examine the immunoprecipitated proteins and inputs.

Cell Culture and Treatment
Human papillomavirus 16–transformed human proximal tubule cells (HK-2, CRL-2190) were purchased from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in a 5% CO2 atmosphere in DMEM/F12 (Gibco; Thermo Fisher Scientific) supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Thirty-six hours after the HK-2 cells were transfected with SIRT1 siRNA or scramble RNA, cisplatin (25 μm; Sigma-Aldrich) was added to the culture medium with or without SP600125 (10 μm; Sigma-Aldrich) for another 14 hours. Then, the cells were harvested for the detection of apoptosis and viability.

Measurement of Cell Viability
HK-2 cells grown in 24-well plates were treated as previously described. Then, cell viability was measured using CCK-8 assay (Cell Counting Kit-8; Dojindo, Japan) and the protocol supplied by the manufacturer. The experiments were performed in four duplicate wells for different treatment groups and repeated three times independently.

SIRT1 Gene Silencing by RNA Interference
Small interference RNA of human SIRT1 gene and scrambled RNA were designed and synthesized by GenePharma Co., Ltd. (Suzhou, China). The SIRT1 siRNA sequences were as follows: SIRT1 5'-CCAUCUCUCUGUCACAATUTT and antisense 5'-AUUUGU-GACAGAGAGAUGCATT, SIRT1 5'-CCAACGCUCUAGGAUAUTT and antisense 5'-AUUACUCUCAUGCGCUUUGTTT, and scrambled RNA sense 5'-UUCUCGGAGAAGUGUGU, and antisense 5'-ACGUGACUGUUCGGAGAATDT. siRNA incubated with Lipofectamine 2000 Transfection Reagent (Invitrogen) was transfected into HK-2 cells for 48 hours, the efficiency of which was detected by immunoblotting for the SIRT1 protein.

Plasmid Constructs
Plasmids of flag-tagged full-length cDNA of human DUSP16 were obtained from Genechem Co., Ltd. (Shanghai, China). In total, seven lysines of potential acetylation site of DUSP16 protein were picked and determined onto the Mouse Array v2.0 (8 K; Arraystar). After having washed the slides, the arrays were scanned with an Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the Limma software package. Differentially expressed mRNAs with statistical significance were identified and annotated according to the KEGG database to locate the most affected signaling pathways. Then, the top 50 distinguished pathways were integrated into the Pathway-Act-Network to elucidate the core signaling pathway.

Statistical Analyses
All values are expressed as mean±SEM and were analyzed with GraphPad Prism 6 using the unpaired Student’s t test or ANOVA with post hoc analysis. The microarray data were analyzed using Fisher exact test. Values of P<0.05 were considered significant.

ACKNOWLEDGMENTS
We thank Huiyong Yin (Shanghai Institutes for Biological Sciences, Chinese Academy of Science) for helping with mice serum creatinine measurement.

The study was supported by National Natural Science Foundation of China grants 31471101, 81520108006, and 81130075 and grants from National Basic Research Program of China 973 program 2012CB517704 and 985 project 985 III-YFX0302.

DISCLOSURES
None.

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Corrigendum

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Kidney Injury in Mice in a SIRT1-Dependent Manner
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J Am Soc Nephrol doi:10.1681/ASN.2016040385. The au-
thors wish to add reference to the following study in which
the significance of nicotinamide adenine dinucleotide
(NAD) biosynthesis to AKI susceptibility and the potential
for NAD precursor supplementation to prevent or treat AKI
were also demonstrated:

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Published online ahead of print. Publication date available at www.jasn.org.