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Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression

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ABSTRACT

NAD^+-dependent protein deacetylase Sirt1 regulates cellular apoptosis. We examined the role of Sirt1 in renal tubular cell apoptosis by using HK-2 cells, proximal tubular cell lines with or without reactive oxygen species (ROS), H_2O_2. Without any ROS, Sirt1 inhibitors enhanced apoptosis and the expression of ROS scavenger, catalase, and Sirt1 overexpression downregulated catalase. When apoptosis was induced with H_2O_2, Sirt1 was upregulated with the concomitant increase in catalase expression. Sirt1 overexpression rescued H_2O_2-induced apoptosis through the upregulation of catalase. H_2O_2 induced the nuclear accumulation of forkhead transcription factor, FoxO3a and the gene silencing of FoxO3a enhanced H_2O_2-induced apoptosis. In conclusion, endogenous Sirt1 maintains cell survival by regulating catalase expression and by preventing the depletion of ROS required for cell survival. In contrast, excess ROS upregulates Sirt1, which activates FoxO3a and catalase leading to rescuing apoptosis. Thus, Sirt1 constitutes a determinant of renal tubular cell apoptosis by regulating cellular ROS levels.

A growing body of evidence has accrued that the balance between growth and death in renal tubular cells plays an important role in determining kidney function [1]. Apoptosis, one of the modes of cell death, participates in kidney physiologic remodeling processes [2] and is thought to contribute to cell loss and structural damage in kidney diseases [3]. For instance, renal proximal tubular cells exhibited apoptosis in streptozotocin-induced diabetic mice [4] as well as in diabetic patients [5], suggesting that tubular apoptosis might precede tubular injuries. Reactive oxygen species (ROS) is involved in the pathogenesis of tubular cell apoptosis in ischemia-reperfusion or toxic acute renal failure [6]. ROS includes superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical. Among them, H_2O_2 is a pathogenic mediator generated during hypoxia/reoxygenation or ischemia-reperfusion injury [7]. Therefore, understanding the signaling pathways of H_2O_2-induced cell apoptosis would provide important clues to the elucidation of the mechanisms of renal tubular cell injury and acute renal failure.

To overcome the ROS generation, cells are equipped with antioxidant defense systems that serve to minimize the susceptibility to ROS. Catalase is one of the antioxidant enzymes which metabolize H_2O_2 and contributes critically to the cellular ROS resistance [8]. The overexpression of catalase showed the elongation of lifespan in mice, showing its positive regulatory role in mammalian aging [9]. Furthermore, conditional transgenic mice overexpressing catalase in proximal renal tubular cells manifested the attenuation of tubular apoptosis and pro-apoptotic gene expression as well as the decrease in interstitial fibrosis [10]. These results clearly showed that catalase played a critical role in the protection of renal tubular cells against apoptosis. On the other hand, accumulating evidence has demonstrated that H_2O_2 or ROS mediated intracellular responses to extracellular stimuli and had a beneficial action [11]. It has been reported that the treatment with antioxidants, either pharmacologically or by the overexpression of endogenous anti-oxidative enzymes, increases apoptosis or stops cell proliferation [12].

One genetic pathway that mediates cell survival or response to ROS stress comprises Sir2, an NAD^+-dependent protein deacetylase and a founding member of the sirtuin family [13]. Sir2 works in a wide array of cellular processes, including gene silencing, longevity, DNA damage repair and cellular apoptosis. Stimulation of Sir2 is sufficient to extend lifespan in yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice [13]. Sirt1, a mammalian ortholog of Sir2, deacetylates many target proteins, such as p53 and forkhead (FoxO) transcription factors, which provides the protection against apoptosis and plays an essential part in mediating

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the survival of a lot of types of the cells. Recent studies have demonstrated that Sirt1 regulates FoxO transcriptional activity by binding and deacetylating FoxO [14]. In mice with cardiac-specific overexpression of Sirt1, Sirt1 protected heart from oxidative stress induced by paraquat with the increased expression of catalase through FoxO-dependent mechanism [15]. Therefore, it is surmised that Sirt1 is an attractive candidate regulating renal cell survival against oxidative stress. However, the effects of Sirt1 on ROS and ROS-induced renal cellular apoptosis have not been fully elucidated thus far.

In the present study, we examined whether Sirt1 had any roles in the basal cellular conditions and whether it exerted a protective action against exogenous oxidative stress in renal tubular cells. We have demonstrated herein that Sirt1 maintains renal cell survival in the unstimulated condition and that in the face of exogenous ROS insults, Sirt1 protects against apoptosis through the induction of anti-oxidative molecule, catalase by a FoxO3a-dependent mechanism.

Materials and methods

Cell culture and materials. HK-2 cells (CRL-2190, American Type Culture Collection, USA) of human proximal tubular origin were grown in keratinocyte serum-free medium plus 5 ng/ml epidermal growth factor and 40 mg/ml bovine pituitary extract (Gibco Laboratories, New York, USA), penicillin 100 IU/ml, and streptomycin 100 μg/ml (Invitrogen, Carlsbad, CA) at 37 °C, 95% air and 5% CO_2. They were plated in 6-well plates and used in the experiments at 80% confluency. Different types of Sirt1 inhibitors, sirtinol and nicotinamide (NAM), were obtained from Sigma (St. Louis, MO). Cytoplasmic and nuclear franctions of HK-2 cells were obtained by using Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL, USA).

H_2O_2-induced injury for HK-2 cells. Confluent monolayers (80%) of HK-2 cells grown in 6-well plates were treated with or without 400 or 800 μmol/l H_2O_2 diluted in serum-free media. Eight hours after the exposure, the cells were washed three times with inhibitor-free medium and harvested for immunoblotting.

Apoptosis measurements. Annexin V-FITC in combination with propiumiodine (PI) was used to quantitatively determine the percentage of cells undergoing apoptosis, as described previously [16]. Briefly, after cells were treated with reagents in the experiments, the monolayer was released by a brief incubation with a Trypsin–EDTA solution. Cells (10^5) were resuspended in 1× binding buffer (BD Pharmingen, San Diego, CA) and incubated with annexin V-FITC for 15 min at room temperature, in the dark, followed by PI staining. Cells were analyzed within 1 h in a FACS Caliber flow cytometer. Cellquest software (Becton Dickinson) was used to analyze the data. Early apoptotic cells were stained with annexin V alone, whereas late apoptotic or necrotic cells were stained with both annexin V and PI.

Constructs and transfection. Sirt1 cDNA was cloned from human kidney cDNA using the primers as described previously [17]. The

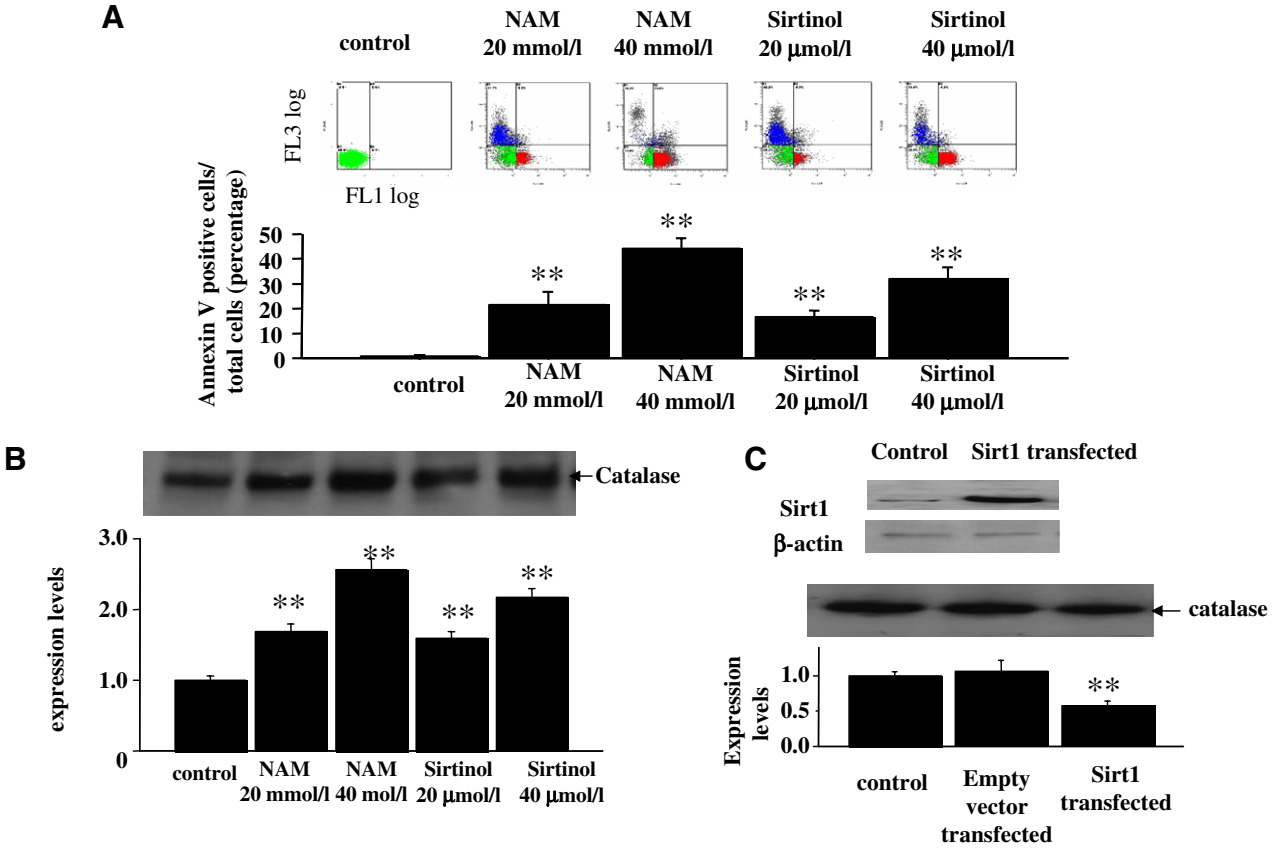

Fig. 1. The role of Sirt1 in cellular survival under physiological condition. (A) HK-2 cells cultured in serum-free conditions were treated with different types of Sirt1 inhibitor, NAM or sirtinol for 48 h. Apoptosis was determined by FACS. Each graph for FACS represents the population of the cells stained with Annexin V (*X*-axis) and propiumiodine (*Y*-axis) (upper pannel). Bar graph summarizing the FACS results are shown in the lower pannel. Data are means ± SE. $^{*}p < 0.05$ vs control. All experiments were conducted three times using different cultures. (B) After the treatment with Sirt1 inhibitors, the cell lysates were immunoblotted with an anti-catalase antibody. Blots were representative for three independent experiments (upper pannel). The bar graph in the lower pannel shows the quantification of the bands intensity. $^{**}p < 0.01$ vs control. (C) Sirt1 expression vectors were transfected in HK-2 cells. Cell extracts were subjected to immunoblot analysis with an anti-SIRT1 antibody (upper pannel) and anti-catalase antibody (lower pannel). The bar graph shows the quantification of the band intensity in immunoblotting for catalase expression. The result is representative band of three independent experiments.

PCR product was subcloned into the mammalian expression vector, pcDNA3.1(+) (Invitrogen). The expression plasmid containing Sirt1 cDNA (pcDNA3.1-Sirt1) and the control plasmid (pcDNA3.1) were transfected into HK-2 cells by using Lipofectamine 2000 (Invitrogen). Forty-eight hours after the transfection, cells were harvested for immunoblotting In addition, Sirt1-transfected HK-2 cells were treated with or without 800 μmol/L H_2O_2 (Sigma–Aldrich) for 8 h.

Knockdown experiment using siRNA. Small interference RNA (siRNA) against FoxO3a and RNAi negative control were provided by Invitrogen. HK-2 cells were transfected with either siRNA (100 pmol/μl) using Lipofectamine2000 according to the manufactures' instructions for 48 h. HK-2 cells transfected with siRNAs were treated with H_2O_2 for 8 h, and harvested for analyzing apoptosis.

Immunoblotting. Immunoblotting was performed as described previously [17], using a mouse anti-Sirt1 antibody, a mouse β-actin antibody (Cell Signaling Technology, Frankfurt, Germany), and a mouse anti-catalase antibody (Sigma–Aldrich). Band intensities were quantified with the Scion Image Software (Scion Corp, Frederick, MD).

Statistics. Data were expressed as the means ± SEM. Data were analyzed using one-way or two-way analysis of variance, as appropriate, followed by a Bonferroni multiple comparison post hoc test. *P*-values less than 0.05 were considered statistically significant.

Results

Sirt1 inhibition induced apoptosis in renal tubular cells under the normal condition

To investigate the function of Sirt1 in the viability of renal tubular cells, we examined the effects of different types of the Sirt1 inhibitor, nicotinamide (NAM) and sirtinol [18]. Treatment with NAM for 48 h caused dose-dependent increases in cell apoptosis (Fig. 1A). Similar findings were obtained with sirtinol. Both NAM and sirtinol upregulated the expression levels of catalase, one of the radical scavengers and of the Sirt1-regulated molecules (Fig. 1B). Next, we constructed Sirt1 overexpression vector using pcDNA3.1 vector (Fig. 1C, upper panel). Overexpression of Sirt1 downregulated the expression levels of catalase (Fig. 1C, lower panel).

H_2O_2 inducesd cellular apoptosis and Sirt1 expression in renal tubular cells

We next investigated the role of Sirt1 under the condition of excessive oxidative stress. Treatment with H_2O_2 induces acute oxidative stress in the cell, which mimics several pathological conditions including ischemic reperfusion injury. As shown in Fig. 2A, the population of the cells stained with annexin V, a marker of apoptotic changes, were increased 8 h after the incubation with H_2O_2 in a dose-dependent manner. Concomitantly, the protein expression of Sirt1 was upregulated by the treatment with H_2O_2 for 8 h (Fig. 2B). These data suggested that Sirt1 had some role in H_2O_2-induced cell apoptosis.

Sirt1 overexpression rescued H_2O_2-induced cellular apoptosis in renal tubular cells

To elucidate the role of Sirt1 in H_2O_2-induced cellular apoptosis, the effects of Sirt1 overexpression were examined. Treatment with H_2O_2 (800 μmol/l) significantly enhanced cellular apoptosis, which was attenuated by Sirt1 overexpression (Fig. 3A). We further evaluated the role of Sirt1 in the regulation of catalase expression by H_2O_2. H_2O_2 induced the upregulation of catalase

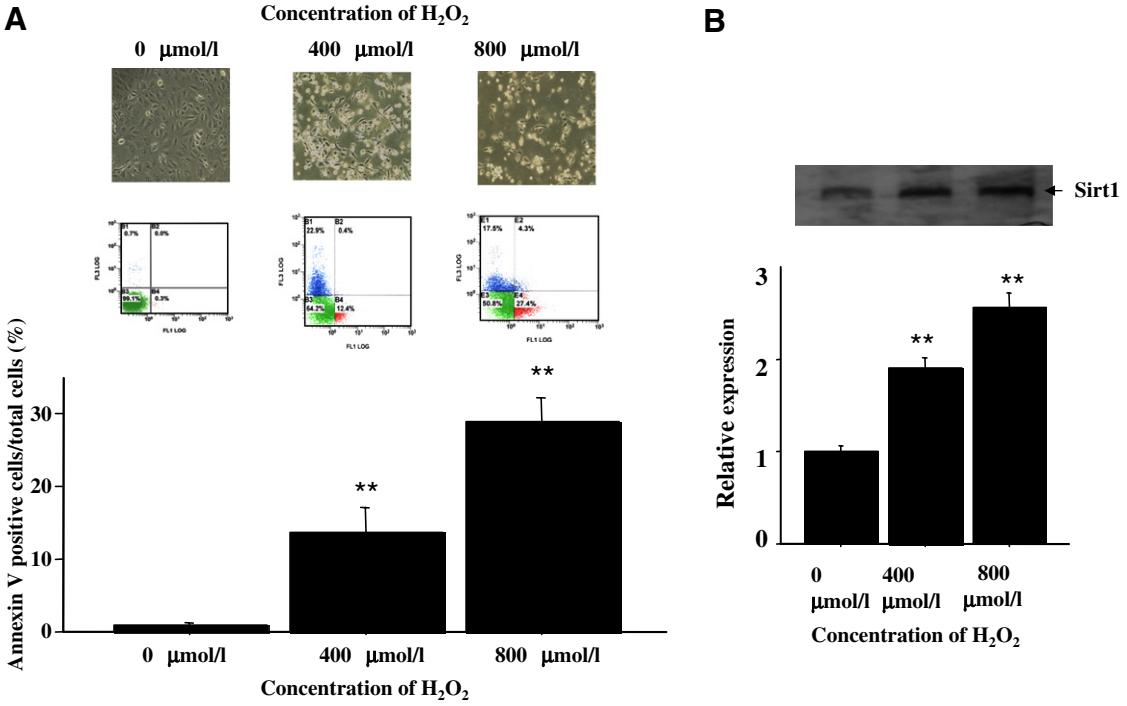

Fig. 2. The role of Sirt1 in cellular survival under H_2O_2-stimulated condition. (A) HK-2 cells were treated with H_2O_2 (400 or 800 μmol/l) for 8 h. Representative microscopic findings of the cell were shown in the upper pannel. After the H_2O_2 treatment, cells were stained with Annexin V and propiumiodine and were subject to FACS. Each graph for FACS represents the population of the cells stained with Annexin V (*X*-axis) and propiumiodine (*Y*-axis) (middle pannel). Bar graph shows the population of Annexin V positive apoptotic cells. Data are means ± SE. $^{*}p < 0.05$ vs control. (B) Sirt1 expression was increased by the stimulation with H_2O_2. Sirt1 expression was detected by immunoblot analysis. Blots was representative for three independent experiments (upper pannel). The bar graph in the lower pannel shows the quantification of the bands intensity. $^{**}p < 0.01$ vs control.

protein, which effect was further enhanced by Sirt1 overexpression (Fig. 3B).

Forkhead transcription factor FoxO3a mediates the cellular survival effects of Sirt1

Sirt1 regulates the catalase expression negatively (Fig. 1C) or positively (Fig. 3B), depending on the underlying condition. To understand this dual regulatory mechanisms by Sirt1, we investigated the role of Forkhead transcription factor, FoxO3a which is deacetylated by Sirt1 and mediates some of Sirt1's effects. We first examined whether Sirt1 and FoxO3a co-localize in the subcellular compartment. Sirt1 was localized in both cytoplasmic and nuclear fraction of HK-2 cells under basal or H_2O_2-treated conditions (Fig. 4A, upper pannel). FoxO3a was present dominantly within the cytoplasm in the absence of H_2O_2. In contrast, H_2O_2 stimulation translocated FoxO3a from the cytoplasmic to the nuclear fraction (Fig. 4A, lower pannel), resulting in co-localization of both Sirt1 and FoxO3a within the nucleus. In addition, silencing FoxO3a expression by siRNA enhanced the H_2O_2-induced cellular apoptosis (Fig. 4B and C). These data indicated that H_2O_2-induced translocation of FoxO3a into the nucleus constituted an important step to the Sirt1-mediated catalase upregulation and anti-apoptotic effects. Without H_2O_2 stimulation, Sirt1 downregulated catalase expression because of the absence of FoxO3a in the nucleus (Supplementary data Fig. 1).

Discussion

In the present study, we have demonstrated that endogenous Sirt1 down-regulates the expression of catalase and maintains the cellular ROS levels in renal tubular cells. Under the condition of excess amount of ROS, however, the expression of Sirt1 is upregulated with the enhancement of catalase expression serving as a scavenger and the resultant acquirement of anti-apoptotic activity. There exists some controversy on the role of Sirt1 in cell apoptotic process. Several studies show anti-apoptotic action whereas other investigations report pro-apoptotic action [19]. The current study therefore unravels both physiological and pathological significance of Sirt1 in ROS-dependent cell survial and apoptosis of renal tubular cells, and further suggests distinct roles of Sirt1, depending on the underlying conditions where renal tubular cells are exposed.

Sirt1 plays a role in a wide variety of processes, including stress resistance, metabolism, differentiation and aging. Although Sirt1 is also involved in the apoptotic process [19], precise roles of Sirt1 remain a matter of debate. Recently, Alcendor et al. [15] reported that moderate overexpression of Sirt1 in the heart showed resistance to oxidative stress and apoptosis, whereas a high level of Sirt1 expression increased them. In mesangial cells, Sirt1 is reported to protect against TGF-β-induced apoptosis through deacetylation of Smad7 [20]. Nevertheless, the functional role of Sirt1 in renal tubular cells under basal condition has not been identified hitherto. In the present study, we have demonstrated that the inhibition of Sirt1 increases apoptosis (Fig. 1). The present study therefore has disclosed a functional role of Sirt1 in renal tubular cells under basal condition and provides a clue to the understanding of Sirt1.

Several lines of studies have identified catalase as one of the important target molecules of Sirt1 for cellular apoptotic effects. Catalase degrades H_2O_2 and regulates the cellular H_2O_2 levels. H_2O_2 is an uncharged molecule and diffuses freely within and between cells. Accumulating evidence has witnessed that H_2O_2 mediates intracellular biological responses to extracellular stimuli. The treatment with excess antioxidants, either pharmacologically or

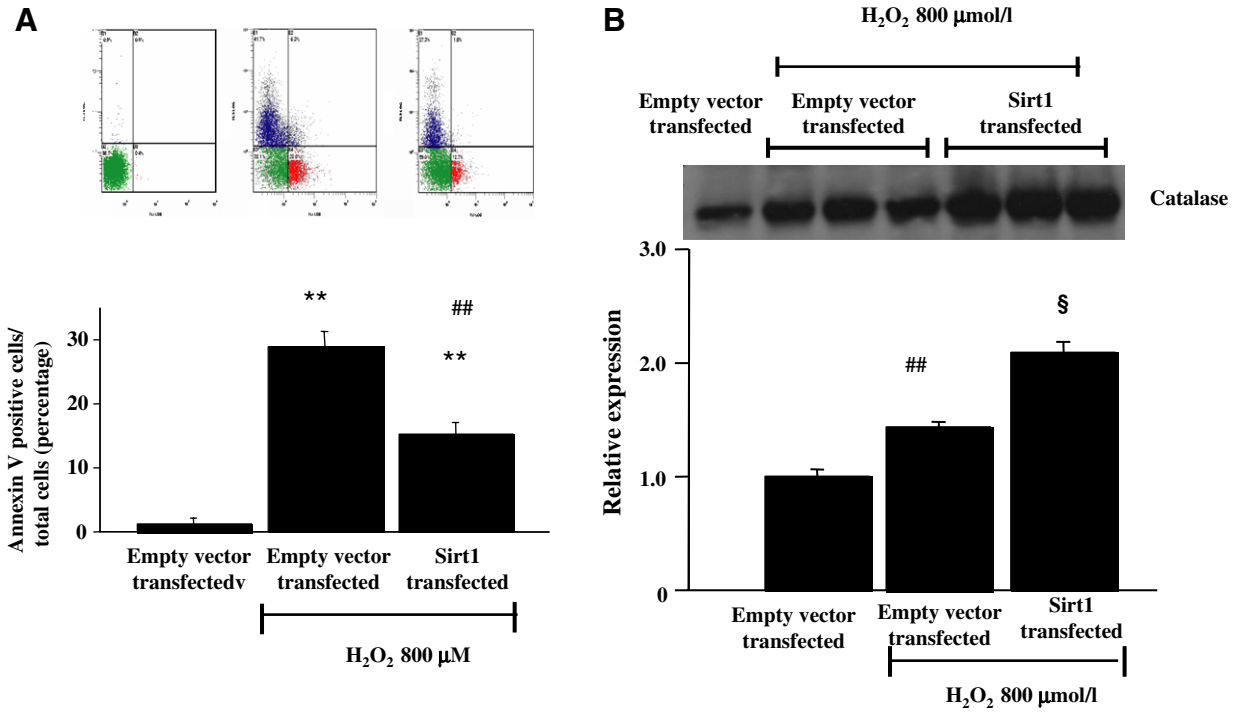

Fig. 3. Sirt1 modulates H_2O_2-induced HK-2 cell apoptosis. (A) After the HK-2 cells were transfected with control or pcDNA3.1 Sirt1 expression vector, cells were incubated with 800 μM of H_2O_2 for 8 h. Cells were stained with Annexin V and propiumiodine and were subject to FACS. Each graph for FACS represents the population of the cells stained with Annexin V (*X*-axis) and propiumiodine (*Y*-axis) (upper pannel). Bar graph summarizing the FACS results showing the population of Annexin V positive apoptotic cells $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs empty vector transfected. (B) After the Sirt1 expression vectors were transfected in HK-2 cells, the cell lysates were immunoblotted with an anti-catalase antibody. Blots was representative for three independent experiments (upper pannel). The bar graph in the lower pannel shows the quantification of the bands intensity. $^{**}p < 0.01$ vs control, $^{\S}p < 0.05$ vs empty vector transfected.

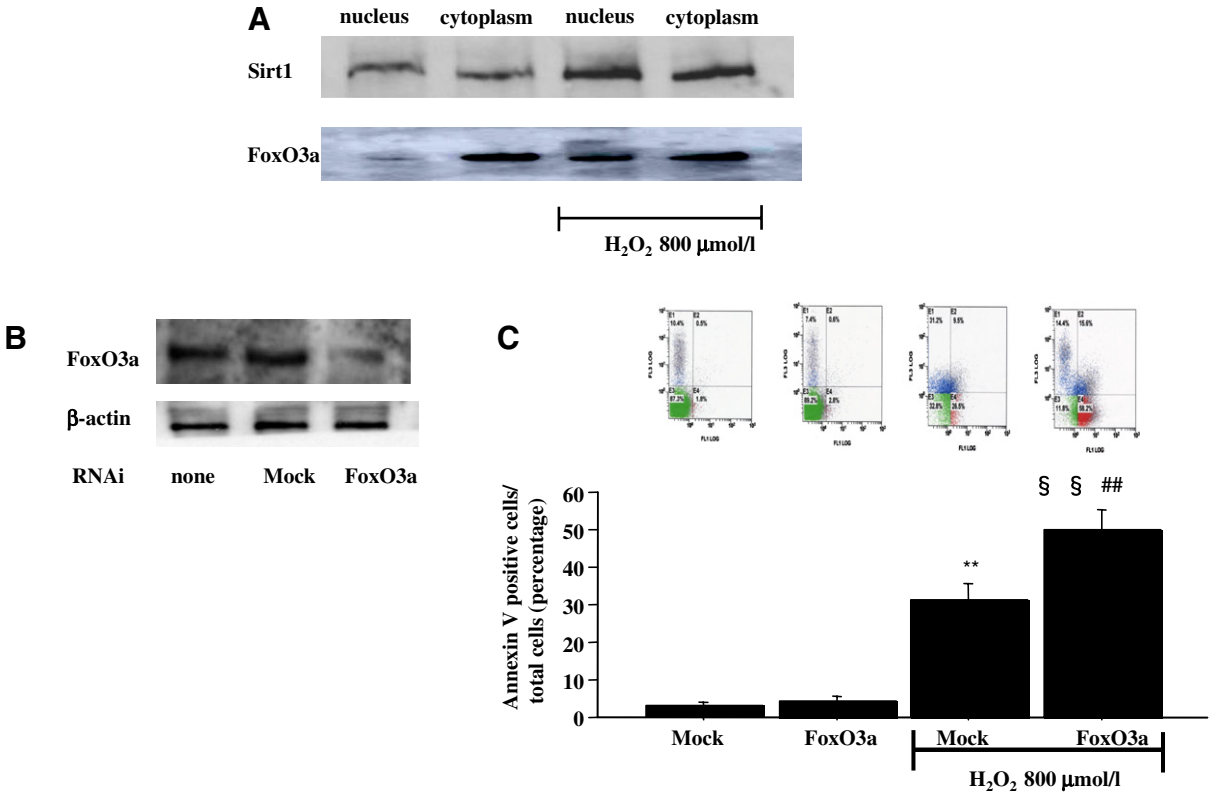

Fig. 4. FoxO3a essential for cell survival against H_2O_2 insults. (A) HK-2 cells with or without the treatment of 800 μmol/l H_2O_2 for 8 h were fractionated and analyzed by immunoblotting analysis with antibodies as indicated. Sirt1 was nuclear and cytoplasmic in the absence of H_2O_2, whereas FoxO3a was mainly in cytoplasmic. In the presence of H_2O_2, intracellular distribution of Sirt1 was not remarkably altered, while FoxO3a was translocated to nucleus. (B) SiRNA silencing FoxO3a expression and negative control (mock) siRNA were transfected (100 pmol/μl) using Lipofectamine2000. The protein expression was examined by immunoblotting and FoxO3a expression was successfully reduced in the cells transfecting FoxO3a siRNA. Blots were representative of three experiments. (C) After HK-2 cells were transfectred with FoxO3a siRNA or mock siRNA, cells were treated with H_2O_2 for 8 h. Cell apoptosis was analyzed by using FACS. H_2O_2-induced apoptosis was markedly enhanced in the cells with FoxO3a siRNA. Without any treatment, FoxO3a siRNA had no effects on cell apoptosis. Each graph for FACS represents the population of the cells stained with Annexin V (*X*-axis) and propiumiodine (*Y*-axis) (upper pannel). Bar graph summarizing the FACS results showing the population of Annexin V positive apoptotic cells (Lower pannel). Data are means ± SE. $^{**}p < 0.01$ vs mock siRNA transfected cells without H_2O_2 stimulation, $^{§§}p < 0.01$ vs FoxO3a siRNA transfected cells without H_2O_2 stimulation, $^{\#\#}p < 0.01$ vs mock siRNA transfected cells with H_2O_2 stimulation.

by overexpression of endogenous anti-oxidative enzymes, increased apoptosis or stopped cell proliferation. Brown et al. [12] also demonstrated that smooth muscle cells overexpressing catalase have enhanced apoptosis. Consistenly, in the present study, Sirt1 inhibition by either NAM or sirtinol upregulated catalase expression, and lead to apoptosis of renal tubular cells (Fig. 1). In concert, these results lend support to the contention that the elimination of physiological levels of H_2O_2 by catalase induces apoptosis.

In contrast to physiological roles of H_2O_2, it has also been demonstrated that excess amount of H_2O_2 increases apoptosis [21]. Brezniceanu et al. [10] recently reported that *db/db* mice overexpressing catalase solely in proximal renal tubular cells exhibited suppressed renal ROS production and reduced tubular apoptosis and pro-apoptotic gene expression. In the present study, we found that the treatment with H_2O_2 upregulated the Sirt1 expression (Fig. 2). Since the overexpression of Sirt1 rescued H_2O_2-induced apoptosis by augmenting catalase expression (Fig. 3), it appears that the upregulation of Sirt1 served as a compensatory mechanism to protect the renal cell against apoptotic pathway. These conflicting actions of H_2O_2 under basal and H_2O_2-stimulated condition may bear on the balance between the levels of the ROS produced from H_2O_2 and antioxidants levels. Thus, although a certain level of oxidant stress favors growth promotion, more severe stress may trigger cell death. Alternatively, excess amount of antioxidants may reduce the ROS level below that necessary for cell survival, precipitating in the apoptotic pathway Taken togerther, in renal tubular cells, Sirt1 controls cellular H_2O_2 levels by regulating the expression of catalase in either a positive or negative manner, and functions as an anti-apoptotic or apototic factor, depending on the ROS status.

In order to elucidate the mechanism for these effects of Sirt1 on cellular apoptosis, we explored the expression of several downstream target molecules of Sirt1. It was reported that the apoptosis of renal tubular cells is linked closely to pro-apoptotic genes, including Bax and Bim [22], and anti-apoptotic genes such as Bcl-2 and Bcl-Xl [23]. In our preliminary study, however, we observed no relationship between Sirt1 and these apoptosis-related molecules (data not shown). In contrast, Sirt1 has been reported to deacetylate not only histones but also nonhistone substrates such as p53, FoxO1, and FoxO3a [15]. By deacetylating these transcrption factors, Sirt1 regulates apoptotic cell death in mice and humans [24]. Indeed, members of FoxO transcription factor, FoxO1 and FoxO3a, enhance ROS resistance [25]. It has also been demonstrated that Sirt1 regulates free radical scavengers, catalase and MnSOD through deacetylation of FoxO1 or FoxO3a [26]. These results imply that under the H_2O_2 insults, the upregulation of Sirt1 modulates FoxO3a activity in the renal tubular cells, which subsequently regulates the expression of catalase [15]. Consistently, our data demonstrated that H_2O_2 stimulation induced the interaction between Sirt1 and FoxO3a and that silencing FoxO3a enhances apoptosis of HK-2 cells (Fig. 4). With the stimulation of excess amount of H_2O_2, Sirt1 upregulated the expression of catalase by utilizing nuclear-translocated FoxO3a. Without any stress, Sirt1 failed to enhance the expression of catalase because of the absence

of FoxO3a in the nucleus (Supplementary Fig. I). The conflicting observations on the action of Sirt1 were attributable to the difference in the intracellular distribution of FoxO3a transcription factors under the different conditions.

In summary, Sirt1 played a protective role in apoptosis in which Sirt1 suppressed the catalase expression under the physiological condition. Under ROS stimulated condition, however, Sirt1 attenuates cellular apoptosis by upregulating catalase through H_2O_2-induced nuclear translocation of FoxO3a. The manipulation of Sirt1 therefore can be a plausible therapeutic target molecule in the treatment of renal tubular damages.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.176.

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