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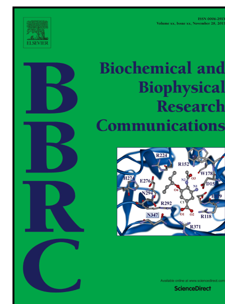


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*Highlights:*

1. MnSOD activity is regulated by SIRT3 high glucose concentrations.

2. MnSOD regulation by SIRT3 protects BRECs from hyperglycemia-induced damage.

3. PARP activation contributes to hyperglycemia-induced SIRT3 downregulation.

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# Deacetylation of MnSOD by PARP-regulated SIRT3 protects retinal capillary endothelial cells from hyperglycemia-induced damage

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**Abstract**

A key initiator in the development of diabetic retinopathy is considered to be the production of reative oxygen species (ROS) in the retinal mitochondria, and their scavenging enzyme, manganese superoxide dismutase (MnSOD), is compromised. However, the mechanism by which high glucose regulates MnSOD is unclear. In this study, we found that a high concentration of glucose inhibited the expression of the histone deacetylase SIRT3, which resulted in a reduction in MnSOD activity in bovine retinal capillary endothelial cells and in the retinas of diabetic rats. Conversely, SIRT3 overexpression attenuated hyperglycemic stress through deacetylation and activationof MnSOD. Furthermore, the hyperglycemia-induced downregulation of SIRT3 involved the activation of poly (ADP-ribose) polymerase (PARP). Our study is the first to link the deacetylation of MnSOD by PARP-regulated SIRT3 with the pathogenesis of diabetic retinopathy. Understanding the role of SIRT3 in the pathogenesis of diabetic retinopathy could help elucidate key molecular targets for future pharmacological interventions.

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*Keywords:* SIRT3; Diabetic retinopathy; PARP; MnSOD; Reactive oxygen species

*Abbreviations:* PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; BREC, bovine retinal capillary endothelial cell

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## 1. Introduction

Diabetic retinopathy is the leading cause of loss of vision in the working population [1-3]. In diabetic complications a key role is played by oxidative stress [4,5], and the generation of mitochondrial superoxide radicals is considered to act as a unifying mechanism that promotes various reactions leading to the development of diabetic complications [6,7]. This condition is further exacerbated by the impairment of the main antioxidant defense mechanisms, including the inactivation of mitochondrion-specific manganese superoxide dismutase (MnSOD) [8]. To date, the regulation of MnSOD levels in diabetes has been mostly reported at the transcriptional level, which occurred in late high glucose [8,9]. By contrast, little is known about whether the level or function of MnSOD is also regulated post-translationally in diabetic retinopathy.

Recently, SIRT3, the Class III $NAD^+$-dependent histone deacetylase, has attracted particular interest with regard to mitochondrial function because it is localized primarily in the mitochondria [11,12]. As the

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primary mitochondrial deacetylase, SIRT3 can regulate energy homeostasis and oxidative metabolism, as well as oxidative stress and cellular injury [13,14]. However, the role of SIRT3 in diabetic vascular complications has not been investigated.

In this study, we demonstrated that SIRT3 downregulation results in a reduction in MnSOD activity in bovine retinal capillary endothelial cell (BRECs) and rat retinas under hyperglycemia, and further that this hyperglycemia-induced SIRT3 downregulation involves the activation of poly (ADP-ribose) polymerase (PARP). We also examined the role of SIRT3 in the control of inflammatory and apoptosis proteins, such as PARP, nuclear factor-κB (NF-κB) and Bax, which have been implicated in various diabetic vascular complications, including diabetic retinopathy.

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## 2. Materials and methods

All chemicals were of reagent-grade quality and were purchased from Sigma Chemicals Co. (St. Louis, MO) unless stated otherwise.

### *2.1. Cell culture and transfection*

Primary cultures of BRECs were prepared as described in our previous study [15]; endothelial cells from passages 3–4 were used in the experiments described below, in which the cells were exposed to either normal glucose (5 mmol/L; NG) or high glucose (30 mmol/L; HG) for 48h.

The Flag-SIRT3 plasmid (Flag-SIRT3) was created by PCR cloning the SIRT3 sequence into the *EcoR*I and *XbaI*I sites of the pcDNA3.0-Flag vector. The SIRT3 primers of SIRT3 were 5′-ATGAATTCATGGCGTTCTGGGGTTGGC-3′ and 5′-ACTCTAGATTTGTCTGGTCCATCAAGCTT-3′. The pcDNA3.0-

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Flag vector (pcDNA) was used as a control empty plasmid. At 24 h after passage, cells were transfected with the plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

The vector pcDNA3.0-HA-MnSOD (MnSOD WT) was created by using specific primers and subcloning the PCR products into the *Kpn*I and *Xbal* I restriction sites of the pcDNA3.0-HA vector. The primers were 5′-ATGTTGAGCCGGGCAGTGTG-3′ and 5′-CTTTTTGCAAGCCATGTATCTTT-3′. The pcDNA3-HA-MnSOD deacetylation mutant (MnSOD K68R) construct was designed to mutate Lys to Arg; the construct was prepared through site-directed mutagenesis based on the wild-type construct by using a QuikChange mutagenesis Kit (Agilent, Carlsbad, CA). The primers used for the one-site mutation (K68R) were 5′-

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GTCACCGAGGAGAGGTACCAGGAGGCG-3′ and 5′-CGCCTCCTGGTACCTCTCCTCGGTGAC-3′. BRECs were transfected with MnSOD siRNA (Si-MnSOD) or control siRNA (Si-NC) by using the siRNA Transfection Reagent according to the manufacturers' instructions (Santa Cruz Biotechnology Inc. USA) for 8 h. Next, the cells were incubated in serum-free medium overnight and then transfected with the constructs of MnSOD WT, Flag-SIRT3, or MnSOD K68R. After 8 h, the cells were left untreated or treated with high glucose, and 48 h later, the cells were harvested and assessed using western blot analysis.

*2.2. Animals*

The animal studies complied with the AAIVE guidelines. We randomly selected 8-week-old male Sprague-Dawley rats (~200 g each; Shanghai Laboratory Animal Center, Chinese Academy of

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Sciences) and intraperitoneally administered either 65 mg/kg streptozotocin (STZ) or citrate buffer alone. Rats were categorized as diabetic when their blood glucose exceeded 16.7 mmol/L at 48 h after STZ administration. Blood glucose levels were 4.6–6.0 mmol/L innondiabetic rats and 18.7–29.8 mmol/L in diabetic rats. For the experimental treatment, SIRT3-overexpressing recombinant lentivirus (Ubi-SIRT3-3FLAG- SV40-EGFP-IRES-puromycin) (LV-SIRT3) and control lentivirus (LV-CON) were obtained from GeneChem (Shanghai, China). The rats were randomized into these 3 treatment groups: (1) normal rats treated with LV-CON ($2 \times 10^8$ TU/mL), (2) diabetic rats treated with LV-CON ($2 \times 10^8$ TU/mL), and (3) diabetic rats treated with LV-SIRT3 ($2 \times 10^8$ TU/mL) by means of subretinal injection [16]. After 8 weeks, the eyes were resected from deeply anesthetized animals and prepared for western blot analysis or

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measurement of in situ ROS generation.

*2.3. Western blotting*

Total protein (50μg/lane) obtained from each sample (BRECs) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked in a 5% nonfat dried-milk solution and incubated overnight with partially purified SIRT3 antibody (LifeSpan BioSciences, Inc. USA), SIRT3 antibody (Cell Signaling Technology, USA), MnSOD antibody (Santa Cruz Biotechnology, Inc. USA), poly(ADP-ribose) (PAR) antibody (R&D Systems, Minneapolis, MN), NF-κB antibody (Cell Signaling Technology), Bax antibody (R&D Systems), Flag antibody (Sigma), or HA antibody (Sigma). Blots were stained with a β-actin antibody

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(Sigma) (as an internal control) to confirm equivalent total protein loading.

*2.4. MnSOD acetylation assay*

Protein lysates were incubated with the MnSOD antibody overnight at 4°C, and then with Protein A/Protein G-coated agarose beads (Millipore, MA) for another 4 h at 4°C. The samples were washed 4 times with ice-cold immunoprecipitation buffer and the supernatants were removed through centrifugation at 2,000 × *g* for 1 min, following which proteins were co-precipitated and eluted from the beads by heating in an immunoprecipitation loading buffer for 15 min at 95°C. The supernatants were collected and subjected to SDS-PAGE and detected using rabbit anti-acetylated lysine antibody (Cell Signaling Technology) or anti-MnSOD antibody.

*2.5. Measurement of intracellular ROS level*

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To measure ROS production, BRECs were incubated (37°C, 30 min) in PBS containing 80 μmol/L of the fluorescent dye 2,7-dichlorofluorescein diacetate (H2DCFDA), and then subjected to flow cytometric analysis.

*2.6 In situ superoxide detection*

Dihydroethidium (DHE; 2 μM, Life Technologies, USA) was used as described previously to detect superoxide in fresh frozen eye sections (10 μm) [17]. Sections were viewed under a fluorescence microscope (excitation, 546 nm; detection, 590 nm), and all images were captured at uniform exposure settings (35 ms). The fluorescence intensity of retinal sections was measured using ImageJ software (National Institutes of Health) [18], and the data were normalized using values from normal rats.

*2.7. Flow cytometry analysis for the measurement of apoptosis*

*measurement*

Apoptosis was assessed by using an Annexin V/propidium iodide (PI) kit according to the manufacturer's instructions (Bender Med Systems, CA). BRECs ($5 \times 10^5$) were washed twice with PBS and suspended in 60 μL of 1× binding buffer. Next, 3.8μL of Annexin V-FITC was added to the cell suspension, vortexed, and incubated for 10 min in the dark, after which 3.8μL of PI was added to the cell suspension and vortexed. Lastly, 60 μL of 1× binding buffer was added, and the samples were evaluated using flow cytometry.

*2.8. Enzyme assays*

The MnSOD activity assay was performed by using the SOD Assay Kit with water-soluble tetrazolium salt (WST-1) as a substrate, as per manufacturer instructions (Dojindo Molecular Technology Inc.) [19].

*2.9. Statistical analysis*

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Group means were compared using one-way ANOVA; for the analyses, we used GraphPad Prism 4.0 software system (GraphPad, San Diego, CA) and the statistical software program SPSS version 17.0 for Windows (SPSS, Chicago, IL). $P < 0.05$ was considered significant in all cases.

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## 3. Results

### *3.1. Effects of hyperglycemia on MnSOD activity, total-protein, and acetylation levels*

ROS activate PARP and cause subsequent ADP ribosylation. PAR is a product of activated PARP and serves as an indicator of PARP activity, which has been shown to be a critical factor in the development of the vascular complications associated with diabetes [15]. Here, treatment with high glucose resulted in an increase in ROS and PAR generation: the levels were higher in the HG group than in the NG group (Figs. 1A and 1B). Moreover, ROS and PAR levels were examined in the retinas of control rats (Norm) and rats that maintained hyperglycemia for 8 weeks (Diab); in the retinas, DHE fluorescence was most intense in the ganglion cell layer (GCL), in association with neovascular tufts, as well as in the inner and outer nuclear layers (INL and ONL). DHE fluorescence was significantly

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higher in the retinas of diabetic rats than in those of normal rats (Fig. 1C). Similarly, the level of PAR was higher in the Diab group than in the Norm group (Fig. 1D). Interestingly, we found no significant difference between the levels of MnSOD expression in retinal endothelial cells incubated with high glucose or normal glucose for 48 h (Fig. 1B), and the expression level was similar in the retinas of diabetic rats and normal rats for 8 weeks (Fig. 1D). Thus, we examined MnSOD activity next.

Analysis of MnSOD enzymatic activity showed that following exposure to high glucose, MnSOD activity was lower than that after exposure to normal glucose (Fig. 1E). Moreover, the results of MnSOD acetylation assays showed that hyperglycemia caused an increase in the level of acetylated MnSOD, but did not alter total MnSOD protein levels (Fig. 1F). We also examined the levels of

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MnSOD activity and acetylation in the Norm and Diab groups. In agreement with the aforementioned results, MnSOD activity was lower in the Diab group than in the Norm group (Fig. 1G), as was the acetylation level of MnSOD, which was evaluated using an immunoprecipitiation assay (Fig. 1H) .

*3.2. Hyperglycemia inhibits SIRT3 expression, which is associated with PARP activation*

Western blot analysis performed using BRECs revealed that the SIRT3 protein level was lower in the HG group than in the NG group (Fig. 2A). Similarly, analysis of the retinas from diabetic rats revealed a reduction in the expression of SIRT3 (Fig. 2B) as compared with that in the retinas from normal rats. Here, to examine how SIRT3 expression is affected when PARP activity was blocked, we used the PARP-specific inhibitor PJ-34; as expected, PJ-34 treatment prevented the downregulation of SIRT3 expression in response to high glucose

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(Fig. 2C).

*3.3. SIRT3 deacetylates and activates MnSOD to scavenge ROS*

To test whether SIRT3 interacts with MnSOD, we overexpressed Flag-tagged SIRT3 in HEK293T cells and immunopurified SIRT3-associated proteins (anti-Flag). Western blotting with an anti-MnSOD antibody revealed the association of MnSOD with SIRT3 (Fig. 3A). Next, to determine whether SIRT3 affects the acetylation status of MnSOD, we overexpressed SIRT3 in BRECs; in these experiments, cells were transfected with pcDNA (control vector) and exposed to normal or high glucose, or transfected with the Flag-SIRT3 expression vector and exposed to high glucose. The results showed that MnSOD acetylation was lower in the SIRT3-overexpressing cells than in the control cells transfected with the empty plasmid (Fig. 3B). Moreover, measurement of MnSOD enzymatic activity in these BRECs revealed

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that the activity was increased following SIRT3 overexpression (Fig. 3C). We also measured ROS levels in the transfected BRECs and found that ROS generation was reduced following SIRT3 over expression (Fig. 3D). Furthermore, we examined the levels of MnSOD acetylation (Fig. 3E) and enzyme activity (Fig. 3F) in the retinas of diabetic rats treated with LV-SIRT3 or LV-CON. As expected, MnSOD was found to be activated through deacetylation after SIRT3 overexpression. In parallel, ROS generation was markedly increased in the retinas of diabetic rats, but this was significantly blunted in LV-SIRT3-treated eyes (Fig. 3G).

*3.4. SIRT3 overexpression inhibits hyperglycemic stress in BRECs and rat retinas by deacetylating MnSOD*

To determine whether MnSOD is a critical target of SIRT3, we constructed plasmids that expressed MnSOD wild-type (MnSOD WT)

or deacetylation mutant (MnSOD K68R). These plasmids and the Flag-SIRT3 construct were used for transfecting BRECs in which endogenous MnSOD expression was knocked down using siRNAs. The knockdown effect of the MnSOD siRNA in BRECs was assessed using western blot analysis (Fig. 4A). Our results showed that exposure to high glucose caused an increase in PAR, NF-κB, and Bax levels in BRECs transfected with wild-type MnSOD, but that the effect was comparatively weaker in BRECs transfected with the MnSOD mutant, and this was similar to what was observed in BRECs co-transfected with wild-type MnSOD and Flag-SIRT3 (Fig. 4B). Consequently, the high-glucose induced increase in apoptosis observed was also suppressed in cells expressing the mutant protein or excess SIRT3 (Fig. 4C).

To establish the role of SIRT3 in protecting the retina against



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hyperglycemic stress, we overexpressed SIRT3 in the retina by using a recombinant lentivirus. Subretinal injection of LV-SIRT3 led to a significant increase in SIRT3 expression in the retina (Fig. 4D), and this LV-SIRT3 treament also suppressed—as compared with LV CON treatment—the increase in NF-κB, Bax, and PAR levels in the retinas of the diabetic rats (Fig. 4D).

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## 4. Discussion

MnSOD is a critical enzyme in the defense against oxidative stress [20]: MnSOD is an evolutionarily conserved protein that is involved in the primary mitochondrial mechanism for eliminating $O_2^-$ or maintaining appropriate levels of $O_2^-$ [21]. Here, we have provided evidence that MnSOD inactivation occurred as a result of the hyperglycemia-induced inhibition of SIRT3 expression in BRECs and retinas of rats. SIRT3 protected the cells against hyperglycemic stress through at least two mechanisms: suppressing the production of the cellular inflammatory molecule NF-κB and attenuating the expression of the gene encoding the apoptosis protein Bax.The overexpressed of SIRT3 was likely involved in the directly activating of MnSOD through deacetylation in retinal endothelial cells, and this subsequently resulted in the inhibition of the mitochondrial ROS/PARP pathway, which is upstream of cellular inflammatory and apoptosis pathways. Furthermore, we showed that the activation of PARP downregulated the expression of SIRT3.

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To our knowledge, we are the first to show that upregulation of MnSOD acetylation was involved in hyperglycemia-induced MnSOD inactivation. Moreover, our results demonstrated that overexpressed SIRT3 could deacetylate and activate MnSOD. Therefore, the role of acetylation and deacetylation of MnSOD in the development of diabetic retinopathy, as it relates to epigenetic changes, should be investigated in future studies.

Mammals and other higher vertebrates express the sirtuin family of $NAD^+$-dependent deacetylases, and these enzymes exhibit distinct subcellular localizations and a wide range of substrate specificities. Previous data linked SIRT3 to the management of ROS produced from mitochondria, which might hold profound implications for aging and late-onset diseases. MnSOD has been consistently shown to be directly deacetylated by SIRT3 in cell-free, in vitro, and in vivo (murine) model systems [22,23]. However, what role SIRT3 plays in diabetic vascular complications is a crucial question that has

remained unanswered to date. In endothelial cells, hyperglycemia can lead to overproduction of mitochondrial ROS, which then results PARP activation and PAR production [15]. The transcription factor NF-κB is a central regulator of inflammatory responses [24], and Bax, a mitochondrial proapoptotic protein of the BCL-2 family, is a marker of mitochondrial stress associated with vascular diabetic complications [25]. We found that SIRT3 overexpression could inhibit hyperglycemic stress, including the generation of ROS, PAR, NF-κB, and Bax. However, SIRT3 might also alter other antioxidant systems. Previously, K68 was demonstrated to be a key acetylation site of MnSOD; when the Lys at this position was changed to an Arg, the acetylation level of the mutant MnSOD was decreased by about 50%, and the enzyme activity was not changed [26]. To determine whether MnSOD is a critical target of acetylation or SIRT3, we generated

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a site-specific mutant individually targeting the lysine and then transfected the MnSOD K68R mutant into BRECs in which endogenous MnSOD expression was knocked down. We found that Lys68 to Arg substitution (MnSOD K68R) resulted in the suppression Of PAR, NF-κB, and Bax generation, which was similar to what was observed in the BRECs that overexpressed SIRT3.

Collectively, the aforementioned results indicate that the effects of MnSOD deacetylation on the suppression of inflammation and apoptosis are mediated by the SIRT3/MnSOD/PARP pathway in retinal endothelial cells after exposure to high glucose concentrations.

SIRT3 appears to be the major deacetylase in mitochondria, but little is known about the mechanism of SIRT3 regulation. Notably, we have reported here that SIRT3 expression was inhibited as a result of the activation of PARP. Because both SIRT3 and PARP are enzymes that use the same cofactor, $NAD^+$, we tested whether SIRT3 inhibition was associated with PARP activation in retinal endothelial cells. The inhibition of PARP activity by PJ-34 upregulated SIRT3 expression,

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suggesting that hyperglycemia-induced PARP activation was at least partly involved in the reduction of SIRT3 expression in these cells. This might create both an amplifying auto-feedback loop that regulates SIRT3 expression and a vicious cycle that further propagates vascular oxidative damage and apoptosis in retinal endothelial cells. However, elucidation of the detailed mechanism will require further investigation under our experimental conditions.

In conclusion, we found that a high concentration of glucose inhibited SIRT3 expression, which resulted in a reduction in MnSOD activity in BRECs and rat retinas, and that PARP activation was involved in the hyperglycemia-induced downregulation of SIRT3. Moreover, SIRT3 overexpression attenuated ROS production and hyperglycemic stress by deacetylating and activating MnSOD. These results suggest for the first time a potential mechanism for SIRT3 regulation of MnSOD activity that is independent of MnSOD expression in the pathogenesis of diabetic retinopathy. Development of treatments targeting SIRT3

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might represent a new avenue of investigation in the diabetes field.

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## Conflict of interest

No potential conflicts of interest relevant to this article were reported.


## Acknowledgments

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**Figure Lengeds**

Fig. 1. (A) ROS generation in BRECs cultured in 5 mmol/L glucose (NG) and 30 mmol/L glucose (HG) together with H2DCFDA. (B) Western blot analysis of PAR and MnSOD expression in cultured BRECs in the NG and HG groups. (C) ROS generation in fresh frozen retinal sections of normal (Norm) and diabetic (Diab) rats, determined through quantitative analysis of DHE fluorescence intensity. (D) Western blot analysis of PAR and MnSOD expression in the retinas of rats in the Norm and Diab groups. (E) Activity of MnSOD in BRECs cultured in 5 mmol/L glucose (NG) and 30 mmol/L glucose (HG). (F) The acetylation of immunoprecipitated MnSOD (MnSOD-ace) expressed in BRECs cultured in NG and HG. (G) Activity of MnSOD in the retinas of normal (Norm) and diabetic (Diab) rats. (H) MnSOD-ace levels in the retinas of Norm and Diab groups.Data shown are means ± SEM. Bars indicate SDs. *$P < 0.05$ vs. NG or Norm; **$P < 0.01$ vs. NG or Norm; ns: $P>0.05$ vs. NG or Norm.

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Fig. 2. Western blot analysis of SIRT3 expression in (A) BRECs cultured in 5 mmol/L glucose (NG) and 30 mmol/L glucose (HG); (B) retinas of normal (Norm) and diabetic (Diab) rats; and (C) BRECs cultured with normal glucose (NG+DMSO), high glucose (HG+DMSO), or high glucose plus the PARP-specific inhibitor PJ- 34 (HG+PJ-34). Data are means ± SEM. Bars indicate SDs. *P < 0.05 vs. NG or Norm or HG+DMSO; **P < 0.01 vs. NG orNorm.

Fig. 3. (A) Interaction between transfected SIRT3 and endogenous MnSOD. HEK293T cells were transfected with the Flag-SIRT3 construct or pcDNA, and proteins were immunoprecipitated, and analyzed through western blotting for SIRT3 association with endogenous MnSOD. Acetylation of immunoprecipitated MnSOD (MnSOD-ace) expressed in BRECs (B), MnSOD enzyme activity (C) profiles and ROS generation (D) in the following cell-treatment groups: normal glucose + pcDNA (NG+pcDNA), high glucose + pcDNA (HG+pcDNA), and high glucose + Flag-SIRT3 overexpression (HG+Flag-SIRT3). Acetylation of immunoprecipitated

MnSOD (MnSOD-ace) expressed in retinas (E), MnSOD enzyme activity (F) and ROS generation (G) in the following rat treatment groups: Norm +LV CON, Diab +LV CON, and Diab + LV SIRT3. Data are means ± SEM. Bars indicate SDs. *P < 0.05 vs. NG+pcDNA or Norm+LV-CON; **P < 0.01 vs. NG+pcDNA or Norm+LV-CON; #P<0.05 vs. HG+pcDNA or Diab+LV-CON; ##P< 0.01 vs. HG+pcDNA or Diab+LV-CON.

Fig. 4. (A) Effect of siRNA-mediated knockdown on MnSOD expression in BRECs incubated in normal glucose. (B) SIRT3 and MnSOD K68R mutant expression lowered the levels of the hyperglycemic stress in BRECs. HA-MnSOD WT, HA-MnSOD K68R, and HA-MnSOD WT + Flag-SIRT3 were expressed in BRECs in which endogenous MnSOD was knocked down using siRNAs. (C) Flow cytometric analysis of apoptosis levels in the 5indicated groups. (D) SIRT3, NF-κB, Bax, and PAR protein expression profiles in the following rat treatment groups: Norm +LV CON, Diab +LV CON, and Diab + LV SIRT3. Data are means ± SEM. Bars indicate SDs. *P < 0.05 HG+Si-MnSOD+HA-MnSOD K68R; ** P < 0.01 vs. HG+Si-

MnSOD+HA-MnSOD K68R or Si-MnSOD or Norm+LVCON; # $P < 0.05$ HG+Si-MnSOD+HA-MnSOD WT+Flag-SIRT3 or Diab+LV-CON; ## $P < 0.01$ HG+Si-MnSOD+HA-MnSOD WT+Flag-SIRT3 or Diab+LV-CON.

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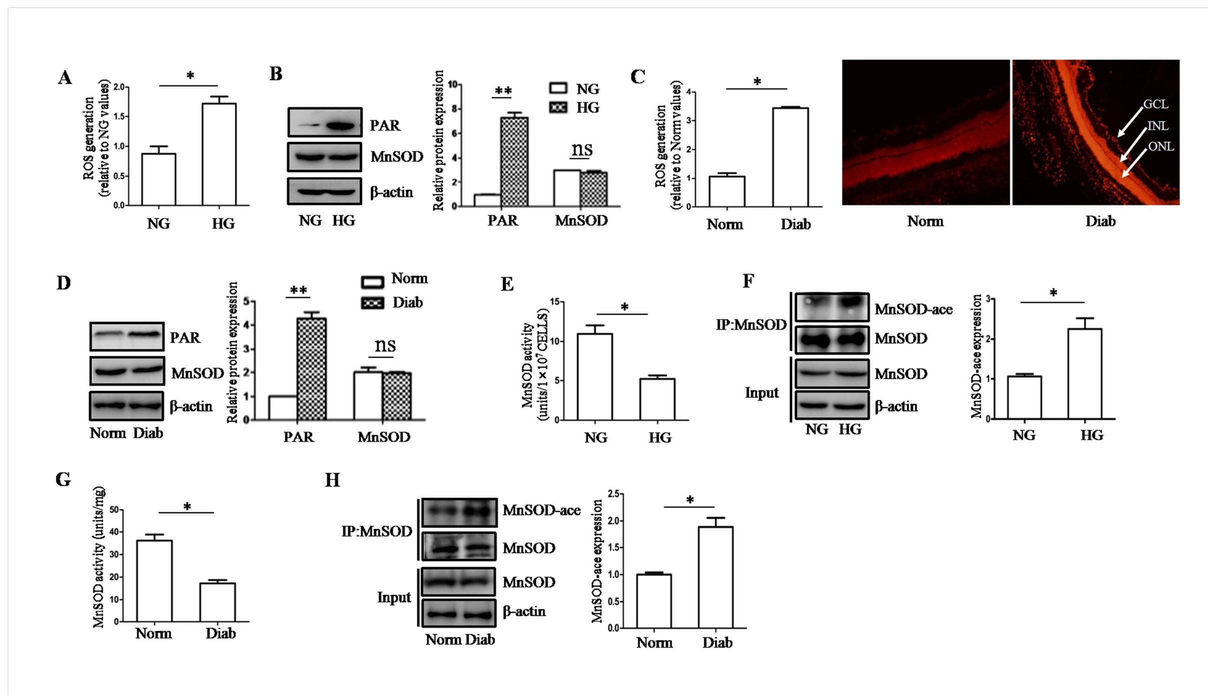


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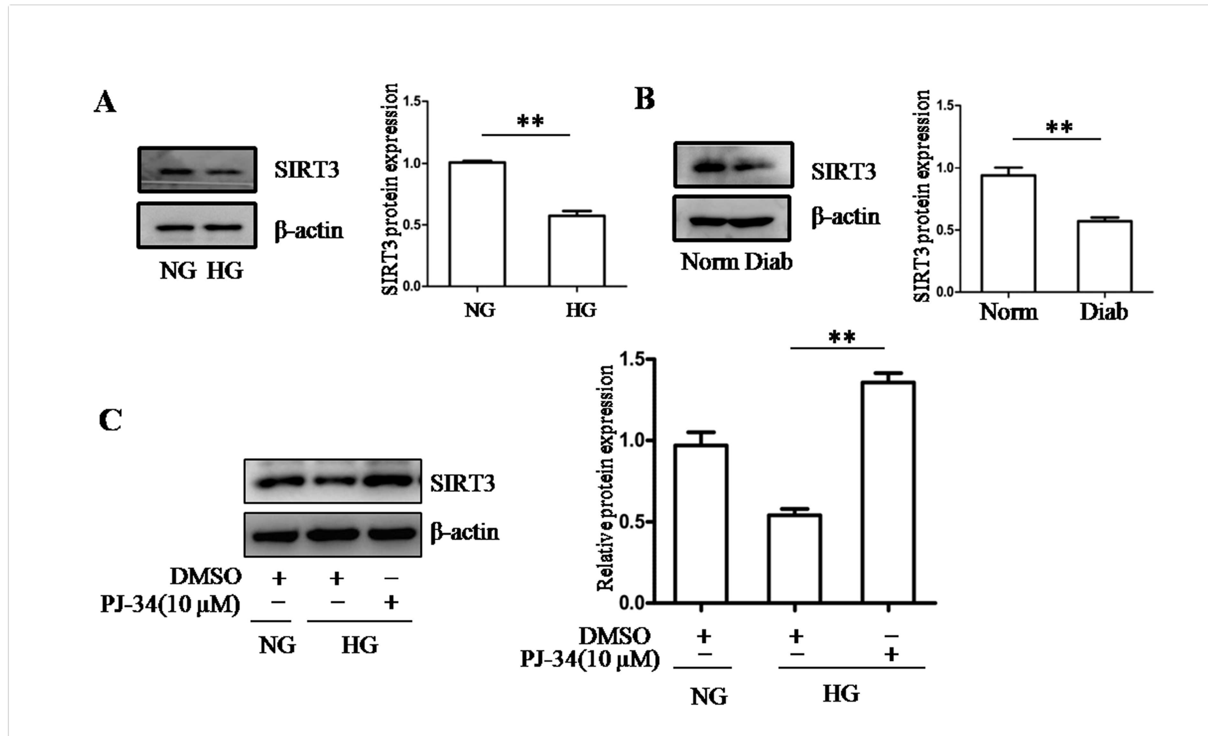


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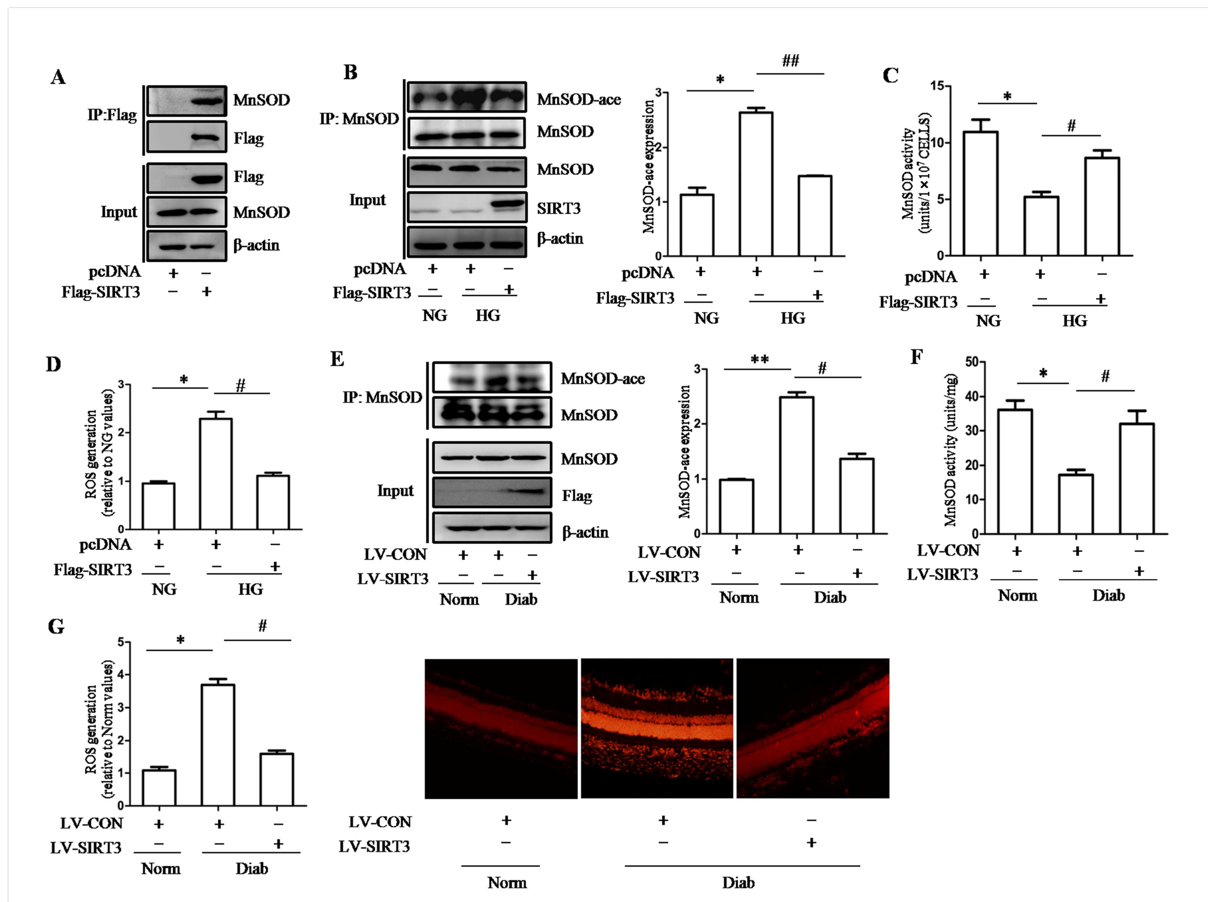

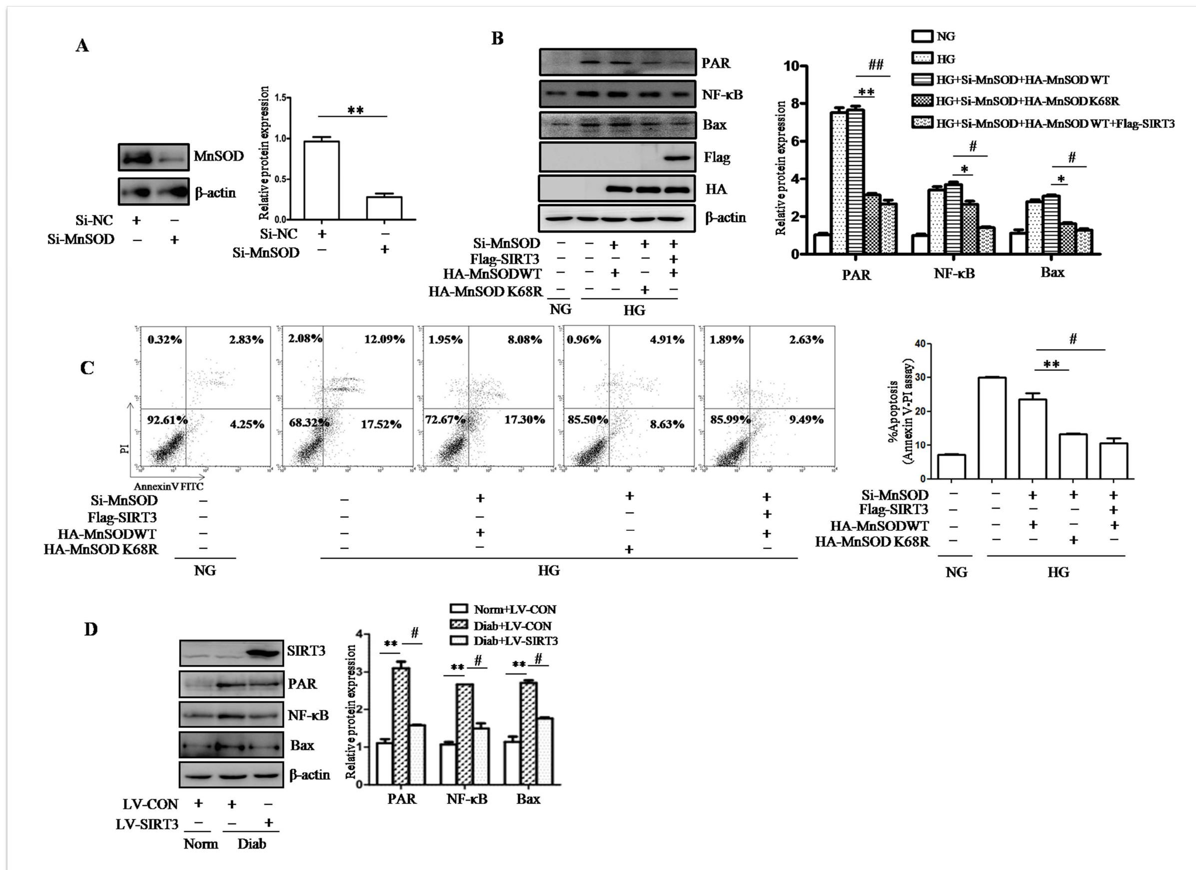


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