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Decreased expression of sirtuin 6 is associated with release of high mobility group box-1 after cerebral ischemia

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ABSTRACT

Sirtuin 6 (SIRT6) belongs to the sirtuin family of NAD$^+$-dependent deacetylases and has been implicated in the regulation of metabolism, inflammation, and aging. Here, we found that SIRT6 was predominantly expressed in neuronal cells throughout the entire brain. Ischemia models using transient middle cerebral artery occlusion in rats and oxygen/glucose deprivation (OGD) in SH-SY5Y neuronal cells showed that ischemia reduced SIRT6 expression and induced the release of high mobility group box-1 (HMGB1) from cell nuclei. The reduced expression of SIRT6 via treatment with SIRT6 siRNA dramatically enhanced the OGD-induced release of HMGB1 in SH-SY5Y cells. Together, our data suggest that SIRT6 may serve as a potential therapeutic target for HMGB1-mediated inflammation after cerebral ischemia.

1. Introduction

Sirtuins are highly conserved mammalian homologs of yeast Sir2 (silent information regulator 2), a NAD$^+$-dependent protein deacetylase. In mammals, seven separate sirtuins (SIRT1–SIRT7) exist, all of which share the catalytic domain with Sir2. These proteins vary in tissue specificity, subcellular localization, and enzymatic activity [1]. SIRT6 is expressed in most murine tissues and found in thymus, muscle and brain with the high levels [2]. In addition to mediating the maintenance of genomic stability, glucose homeostasis, and aging [2–4], SIRT6 also is involved in inflammation. Specifically, SIRT6 directly interacts with the nuclear factor-κB (NF-κB) RelA subunit or c-JUN and deacetylates histone H3 lysine 9 (H3K9) at NF-κB and c-JUN target gene promoters to suppress proinflammatory gene expression [2,4–6]. In the brain, SIRT6 has emerged as a key histone deacetylase that modulates chromatin structure and gene activity [7], though little is known regarding the expression patterns and function of SIRT6 in pathological condition of the brain.

The non-histone DNA binding protein, HMGB1, is known to act as a pro-inflammatory cytokine in the ischemic brain [8,9]. Immunohistochemistry studies of the cortex and striatum in normal rats demonstrated that HMGB1 is mainly expressed in the nuclei of neuronal cells [10]. After brain ischemia, HMGB1 was found to be translocated from the nucleus to cytoplasm, with the cytoplasmic HMGB1 later gradually released into the extracellular space [8,9]. After binding to receptors expressed in neuronal, glial and endothelial cells, HMGB1 has induced inflammatory cytokines, including tumor necrosis factor-α and inducible nitric oxide synthase [8,11,12]. Although the exact signaling pathways by which brain ischemia induces HMGB1 release have not been clearly defined, post-translational modifications, including phosphorylation, methylation, and acetylation, are thought to regulate active HMGB1 release [13].

Inflammation is one of the key mechanisms mediating brain injury after cerebral ischemia. In this regard, SIRT6 may play a role in cerebral ischemia, given its anti-inflammatory effect in other organs [5,6]. Moreover, as protein deacetylases represent the underlying release mechanism for HMGB1, the protein deacetylase, SIRT6, may also contribute to the extracellular release of HMGB1 in the setting of cerebral ischemia. To address the role of SIRT6 in brain ischemia, we used rat transient middle cerebral artery occlusion (MCAO) model that is the most widely used experimental stroke model.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; GFAP, glial fibrillary acidic protein; HMGB1, high-mobility group box-1; H3K9, histone H3 lysine 9; MCAO, middle cerebral artery occlusion; NF-κB, nuclear factor-κB; OGD, oxygen/glucose deprivation; PBS, phosphate-buffered saline; PI, propidium iodide; RECA-1, rat endothelial cell antigen-1; Sir2, silent information regulator 2; SIRT, sirtuin.

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Herein we describe the expression pattern of SIRT6 in the normal and ischemic brain, as well as detail the role of SIRT6 in HMGB1 translocation after episodes of cerebral ischemia.

2. Materials and methods

2.1. Middle cerebral artery occlusion model

All experiments described in this protocol were approved by the Animal Experiment Committee of Yonsei University College of Medicine and conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were housed in a temperature-controlled animal facility under a 12/12 h reversed light and dark cycle and given with a free access to food and water. MCAO was performed in 8–9-week-old male Wistar rats (Orientbio Inc., Seongnam, Republic of Korea) weighing 280–310 g as previously described [14]. Briefly, the animals were anesthetized with isoflurane (5% for induction and 2% for maintenance) in a mixture of 70% nitrous oxide and 30% oxygen. During operative procedures, body temperature was monitored continuously with a rectal probe and maintained at 37.0 ± 0.2 °C using a homeothermic blanket control unit and a heating pad (Harvard Apparatus, Holliston, MA, USA). A silicone-coated 4-0 nylon monofilament (403556PK10, Doccol Corp., Redlands, CA, USA) was inserted into the left internal carotid artery to occlude the left MCA origin. After 2 h of MCAO, reperfusion was achieved by removal of the monofilament. After 22 h of reperfusion, the rats were sacrificed via transcardial cold saline perfusion under anesthesia using intraperitoneal urethane injection, and then decapitated. The resulting brains were sectioned into 2-mm thick coronal blocks using rat brain matrix, embedded in OCT compound (Sakura Finetek Inc., Torrance, CA, USA), quick-frozen with dry-ice cooled 2-methylbutan, then stored at −80 °C. To examine the effect of ischemia on SIRT6 expression in certain region of brain, the fourth block was used for immunostaining and protein/RNA isolation. For the paraffin blocks, the 2 mm-thick brain blocks were fixed in 4% paraformaldehyde overnight and then embedded in paraffin.

2.2. Immunostaining

Immunohistochemistry was performed for SIRT6, glial fibrillary acidic protein (GFAP), and NeuN, using 4-μm thick paraffin sections. After deparaffinization, antigen retrieval was performed in epitope retrieval solution via an IHC-Tek™ epitope retrieval steamer (IHC world, Woodstock, MD, USA). To stain for HMGB1 or double-stain SIRT6 as well as rat endothelial cell antigen-1 (RECA-1), 10-μm frozen tissues sections were used. The sections were fixed in 4% paraformaldehyde, treated with blocking solution [phosphate-buffered saline (PBS) containing 10% normal goat serum and 1% bovine serum albumin] for 1 h, and then incubated with the primary antibodies at 4 °C overnight. Specifically, the primary antibodies used were rabbit polyclonal anti-SIRT6 (1:300, Novus Biological, Littleton, CO, USA), rabbit polyclonal anti-HMGB1 (1:100, Abcam, Cambridge, Cambridgeshire, UK), goat polyclonal anti-GFAP (1:200, Santa Cruz biotechnology Inc., Santa Cruz, CA, USA), the mouse monoclonal anti-NeuN (1:100, Chemicon, Temecula, CA, USA), and the mouse monoclonal anti-RECA-1 (1:10, Abcam). The tissues were further processed with an avidin–biotin–horseradish peroxidase complex (Vector Laboratories Ltd., Peterborough, Cambridgeshire, UK) for immunohistochemistry or incubated in FITC-labeled anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) and either Cy3-labeled anti-goat IgG (Jackson Immunoresearch Laboratories) or Cy3-labeled anti-mouse IgG (Jackson Immunoresearch Laboratories) for immunofluorescence. The peroxidase signals were developed using a 0.01% 3,3′-diaminobenzidine solution (Vector Laboratories Ltd.), counterstained with hematoxylin, and mounted with permount mounting medium (Fisher Scientific, Pittsburgh, PA, USA). The immunofluorescence-stained sections were mounted in ProLong antifade reagent (Invitrogen) containing 4′,6-diamidino-2-phenylindole (DAPI).

2.3. Counting of SIRT6 positive cells

All images were obtained with an Axio microscope (Carl Zeiss, Inc., Oberkochen, Germany). The percentage of SIRT6 positive cells were estimated by stereological analysis with Stereo Investigator software (MBF Bioscience, Williston, VT, USA) using an optical fractionator. To obtain an unbiased number of SIRT6 positive cells, all contours around the cerebral cortex or caudate putamen were traced under 5× magnification. Counting frame size was set at 80 × 80 μm, and the sampling grid size at 250 × 250 μm. Random placement of the counting frames was accomplished using the motorized stage of microscope controlled by the software. The numbers of total cells and SIRT6 positive cells in the counting frame were counted at 400× magnification by a blinded observer.

2.4. Cell culture and treatments

SH-SY5Y cells and 293T cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 50 units/ml penicillin, and 50 μg/ml streptomycin. For all knockdown experiments, SIRT6 siRNAs were obtained from Bioneer (Daejeon, Republic of Korea) and transfected into the cells using Lipofectamine RNAiMax (Invitrogen). The sequences for the SIRT6 siRNAs were as follows: A siRNA (5′-CGAGGAUGUCGGUGAAUUA-3′), B siRNA (5′-GGAACAUGUUUGUGGAAGA-3′), and C siRNA (5′-CUGGUCUCCAGCUUAAACA-3′). To perform OGD on the SH-SY5Y cells, cells (4×10^5 cells) were plated into a 35 mm dish. After 24 h, these cells were then placed in an anaerobic chamber (Thermo Forma Inc., Waltham, MA, USA), and the medium was replaced with 1 ml of pre-warmed (37 °C) glucose free DMEM previously bubbled with an anaerobic gas mix (95% N_2 and 5% CO_2) for 30 min to remove any residual oxygen. This conditioned medium was then collected after the indicated period of incubation, and the cell debris was removed by centrifugation.

2.5. Real-time RT-PCR

The total amount of RNA was isolated from both ischemic brain tissue and SH-SY5Y cells via an RNeasy Plus kit (Qiagen Inc., Valencia, CA, USA). All quantitative real-time PCR reactions were performed using the SYBR Green reagent (Roche Applied Science, Penzberg, Bavaria, Germany) and a LightCycler 480 System (Roche Applied Science), as per the manufacturers' instructions. The primers used for the quantitative real-time PCR reactions were as follows: rat SIRT2 (5′-TGTCAACACCTCCTGTGGAA-3′ and 5′-TGAGGAGGTCCACCTTTGAG-3′), rat SIRT6 (5′-GGCTACGTGGATGAGGTGAT-3′ and 5′-GGCTTGGGCTTATAGGAACC-3′), rat β-actin (5′-TTCAACACCCCAGCCATGT-3′ and 5′-CAGAGGCATACAGGGACAACAC-3′), human SIRT6 (5′-CCAAGTTCGACACCACCTTT-3′ and 5′-CGGACGTACTGCGTCTTACA-3′), and human GAPDH (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′). All PCR reactions were performed in triplicate, with the relative expression level of mRNA normalized to the β-actin or GAPDH level using the comparative CT (ΔΔCT) method.

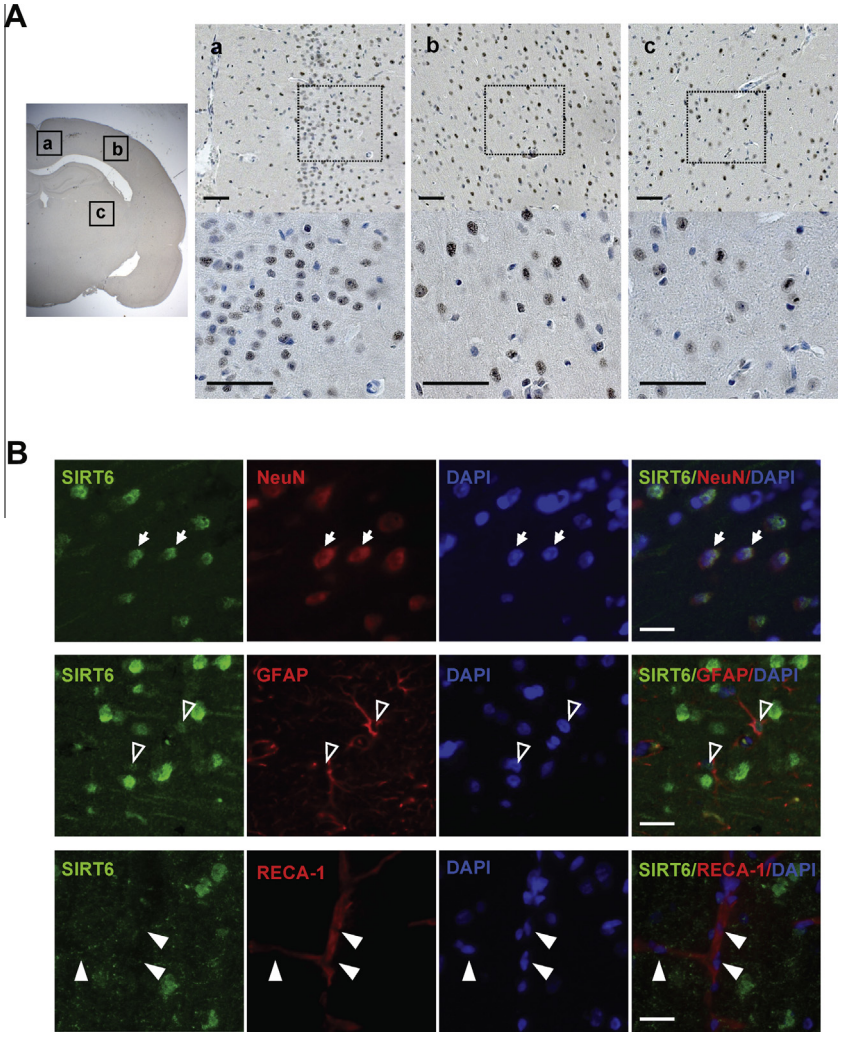

Fig. 1. Expression patterns of SIRT6 in rat brain sections. (A) The coronal section of rat brain was immunostained with anti-SIRT6 antibody. Nuclei were counterstained with hematoxylin. The lettered boxes correspond to the regions used for the photographs. The boxes in "a", "b", and "c" indicate the fields shown at a higher magnification, with the higher magnification views of the regions placed at the bottom of the micrographs. The SIRT6 positive cells are widely distributed in the cortex (a and b) and thalamus (c). Scale bars represent 50 μm. (B) Double immunofluorescence staining was performed using anti-SIRT6 antibody and antibodies specific for neuron (NeuN), astrocytes (GFAP), and endothelial cells (RECA-1). The strong immunoreactivity of SIRT6 was observed in the NeuN-positive neuronal cells (arrows), whereas it was detected at low levels or not detected in GFAP-(open arrowheads) and RECA-1-positive cells (closed arrowheads). DAPI staining was used to demonstrate cellular nuclei. All images were obtained from the cerebral cortex of the rat brain. Scale bars represent 20 μm.

2.6. Western blot analysis

The total protein was isolated from ischemic and nonischemic brain hemispheres. The brain tissues were homogenized in cold radio immunoprecipitation assay buffer with protease inhibitors cocktail (Roche Applied Science). A total of 50 μg of each protein sample was loaded into the SDS–PAGE and transferred into polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc.). The blocked membranes were incubated with anti-HMGB1 (Abcam, Cambridge, Cambridgeshire, UK), anti-SIRT2 (Cell Signaling technology Inc., Danvers, MA, USA), anti-SIRT6 (Abcam for rat SIRT6 and Cell Signaling technology Inc. for human SIRT6), and anti-β-actin (Santa Cruz biotechnology Inc., Santa Cruz, CA, USA) antibodies. All immunoreactive bands were visualized by chemiluminescence using the ECL reagents (GE Healthcare, Fairfield, CT, USA).

2.7. Cell death assay

Cell death was detected by Annexin-V and propidium iodide (PI) staining for necrotic and apoptotic cells. Single cell suspensions were washed in PBS, resuspended in binding buffer, and incubated with FITC-conjugated Annexin-V and PI, as per the manufacturer's instructions (Biobud, Seongnam, Republic of Korea). Cell death was analyzed by flow cytometry (LSRII, BD Bioscience, San Jose, CA, USA).

2.8. Statistical analyses

All statistical analyses were performed using SPSS statistics for windows (version 20.0, IBM Inc., Chicago, IL, USA). Data were presented as the mean ± SD and compared using a two-tailed *t*-test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. High levels of SIRT6 expression in neuronal cells of the rat brain

After immunohistochemical staining, SIRT6 positive cells were found to be widely distributed throughout the entire brain, including cortex, thalamus and striatum (Figs. 1A and 2D). Although SIRT6 positive cells were detected in most of regions of the brain,

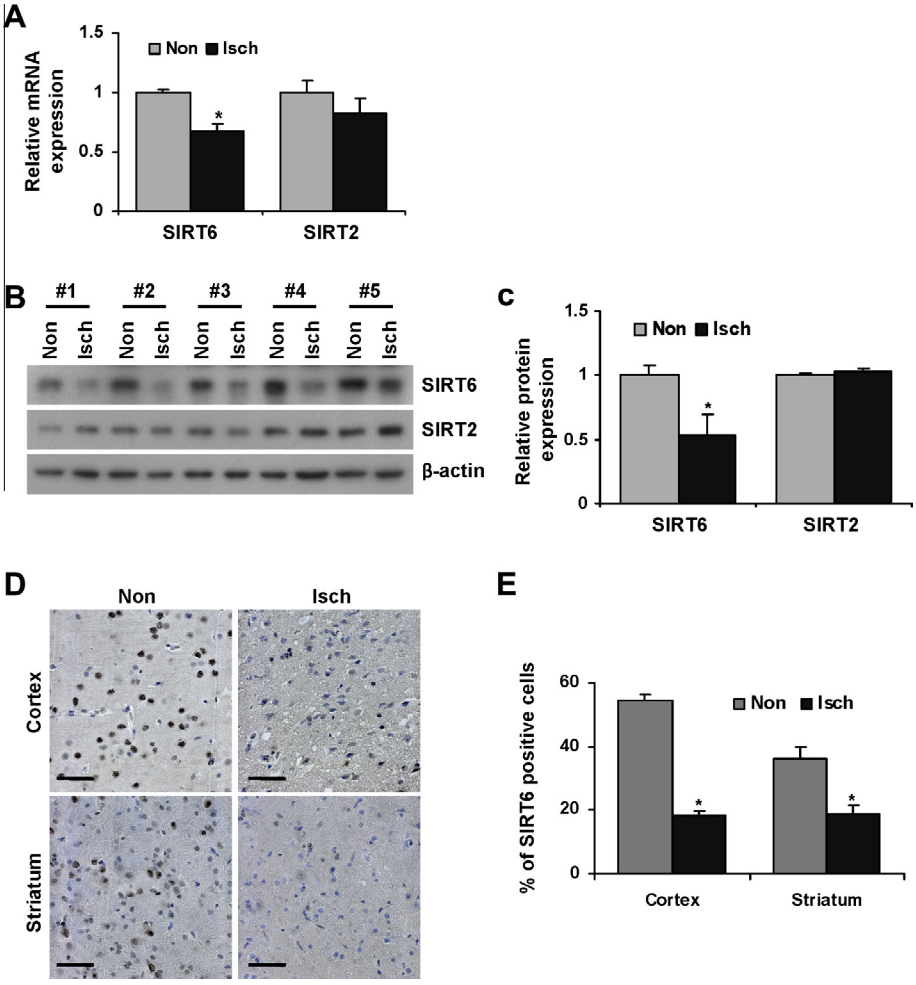

Fig. 2. The effect of ischemic injury on the expression of SIRT6 in brain tissues. (A) The SIRT6 mRNA was reduced in the ischemic region (Isch) of the brain, compared with the nonischemic control region (Non). The mRNA expression of SIRT6 and SIRT2 were analyzed using real-time RT-PCR. The expression levels were normalized with the respect to those of β-actin. Data are expressed as mean ± SD. $^{*}p < 0.01$ vs. nonischemic region, $n = 3$. (B) The SIRT6 protein levels were downregulated in the ischemic regions of brains. Immunoblot analysis of SIRT6 and SIRT2 expressions was performed in the nonischemic (Non) and ischemic (Isch) brain tissues. β-Actin was used as internal control for calculating the fold of change. (C) Image J analysis of the immunoblot in (B). The levels of SIRT6 and SIRT2 proteins were normalized to actin. Data are expressed as mean ± SD. $^{*}p < 0.01$ vs. nonischemic region, $n = 5$. (D) Coronal sections of ischemic brain tissue were immunostained with anti-SIRT6 antibody. SIRT6 positive cells are present in nonischemic cortex and striatum, but rarely found in the infarct areas. Scale bars represent 50 μm. (E) The percentage of SIRT6 positive cells was estimated in the nonischemic (Non) and ischemic (Isch) regions of cortex and striatum by stereological analysis with Stereo Investigator software. $^{*}p < 0.01$ vs. nonischemic region, $n = 3$.

not all cell types were SIRT6 positive. On double immunofluorescence staining [using anti-SIRT6 antibodies in addition to anti-NeuN (neurons), anti-GFAP (astrocytes) or anti-RECA-1 (endothelial cells) antibodies)], most NeuN-positive cells expressed SIRT6 at high levels (Fig. 1B), suggesting that SIRT6 is important in neuronal cells. SIRT6 staining was very weak in GFAP positive cells and not detectable in endothelial cells.

3.2. The decreased expression of SIRT6 after cerebral ischemia

The effect of ischemic injury on SIRT6 expression in the brain was also examined. We found that SIRT6 mRNA levels were significantly decreased in ischemic hemispheres when compared with corresponding unaffected nonischemic regions (Fig. 2A). We further confirmed this reduction in SIRT6 protein levels within the ischemic brain via Western blot analysis (Fig. 2B and C). The expression of SIRT2, a cytoplasmic protein expressed in oligodendrocytes and Schwann cells [15], was not affected by ischemic injury, suggesting that SIRT6 expression levels are much more sensitive to ischemia.

Immunohistochemical staining also demonstrated that SIRT6 expression was significantly decreased in the ischemic cortex and striatum, when compared with nonischemic regions (Fig. 2D and E). The percentage of SIRT6 positive cells was 18.1 ± 1.4% in the cortex of the ischemic hemisphere vs. 54.5 ± 2.0% in the corresponding regions of the non-ischemic hemisphere ($p < 0.01$) (Fig. 2E). Double immunofluorescence staining using anti-SIRT6 and anti-NeuN antibodies also showed that SIRT6 expression was dramatically reduced in neuronal cells within the ischemic region (Fig. 3A).

To determine whether the subcellular localization of HMGB1 was altered in the ischemic brain, tissues were stained with anti-HMGB1 antibodies. Nuclear HMGB1 was found to be translocated into the cytoplasm within the ischemic regions of the brain (Fig. 3B).

3.3. The down-regulation of SIRT6 expression enhances the OGD-induced release of HMGB1 in SH-SY5Y cells

We next evaluated the effect of ischemia on SIRT6 expression and HMGB1 release using *in vitro* ischemia model. SH-SY5Y cells

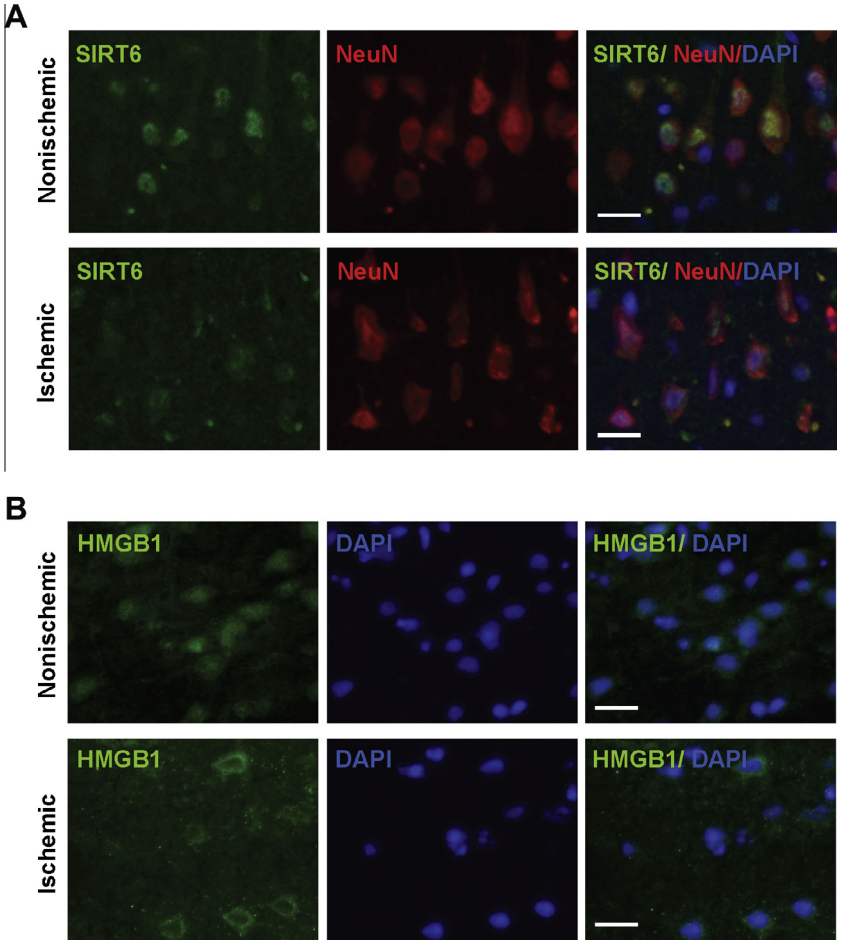

Fig. 3. Decreased SIRT6 expression and HMGB1 translocation from the cell nuclei to cytoplasm during brain ischemia. (A) Double immunostaining using anti-SIRT6 and anti-NeuN antibodies. SIRT6 expression was significantly diminished in neuronal cells within the infarct area. DAPI was used to stain cell nuclei. (B) HMGB1 localization was visualized by double staining with anti-HMGB1 antibody and DAPI. Translocation of HMGB1 from the cell nucleus to cytoplasm was observed in ischemic brain tissues.

were exposed to OGD for 3, 6, and 12 h. SIRT6 expression was significantly reduced as early as 3 h after exposure to OGD (Fig. 4A). The accumulation of the released HMGB1 was also detected starting at 3 h after OGD exposure (Fig. 4B).

To examine whether the reduced SIRT6 expression was associated with the release of HMGB1, we designed three different SIRT6 siRNAs. The knockdown efficiency of the SIRT6 expression by siRNA was evaluated by RT-PCR and Western blot analysis (Fig. 4C and D). The knockdown of SIRT6 in SH-SY5Y cells did not affect OGD-induced cell death (Fig. 4E). However, when the cells treated with SIRT6 siRNA were exposed to OGD, the release of HMGB1 was markedly enhanced when compared with control siRNA-treated cells (Fig. 4F).

4. Discussion

Herein, we demonstrate that SIRT6 is predominantly expressed in neurons in the normal rat brain. Most sirtuins are highly expressed in the human brain at RNA levels [16]. However, their expression patterns including regional distribution and cell type-specific expression in the brain have not been well characterized, with the exception of SIRT1 and SIRT2. Specifically, SIRT1 is ubiquitously expressed across the entirety of both the rodent and human brain, and is predominantly localized in neuronal nuclei [17]. SIRT2 is mainly present in the cytoplasm of oligodendrocytes and Schwann cells that form the myelin sheaths and axons, respectively [18]. Our findings suggest that SIRT6 is similar in distribution pattern and cellular localization as SIRT1.

SIRT6 expression was found to decrease in ischemic areas of the rat brain as well as following OGD exposure in SH-SY5Y cells. SIRT6 knockdown by siRNA treatment robustly enhanced the release of HMGB1 in SH-SY5Y cells exposed to OGD. These findings suggest that SIRT6 is somehow linked with the release of HMGB1 which is known to mediate ischemic injury in the brain [9,11,19]. Mechanisms of HMGB1 release include acetylation of HMGB1 [20], acetylation of chromatin [21], and NF-κB activation [22]. Hyperacetylation on lysine residues of HMGB1 is known as a major mechanism by which pro-inflammatory stimulus induces HMGB1 release in activated monocytes [20]. Our biochemical analysis using co-immunoprecipitation assay demonstrates that SIRT6 did not directly interact with HMGB1 (data not shown), suggesting that HMGB1 is not direct substrate of SIRT6 and acetylation levels of HMGB1 may not be affected by SIRT6. Moreover, the other group did not observe the hyperacetylated HMGB1 after cerebral ischemia using two-dimensional electrophoresis [9].

The chromatin acetylation status is also important in altering the localization of HMGB1. Treatment with trichostatin A, a general histone deacetylase inhibitor, suppresses HMGB1 binding to chromatin, suggesting that hypoacetylation of one or more components of chromatin favors HMGB1 binding [21]. Given that SIRT6 is a major histone deacetylase in the brain [7], it is possible that SIRT6 is involved in the maintaining the nuclear localization of HMGB1 through modifications in the chromatin structure. In a septic

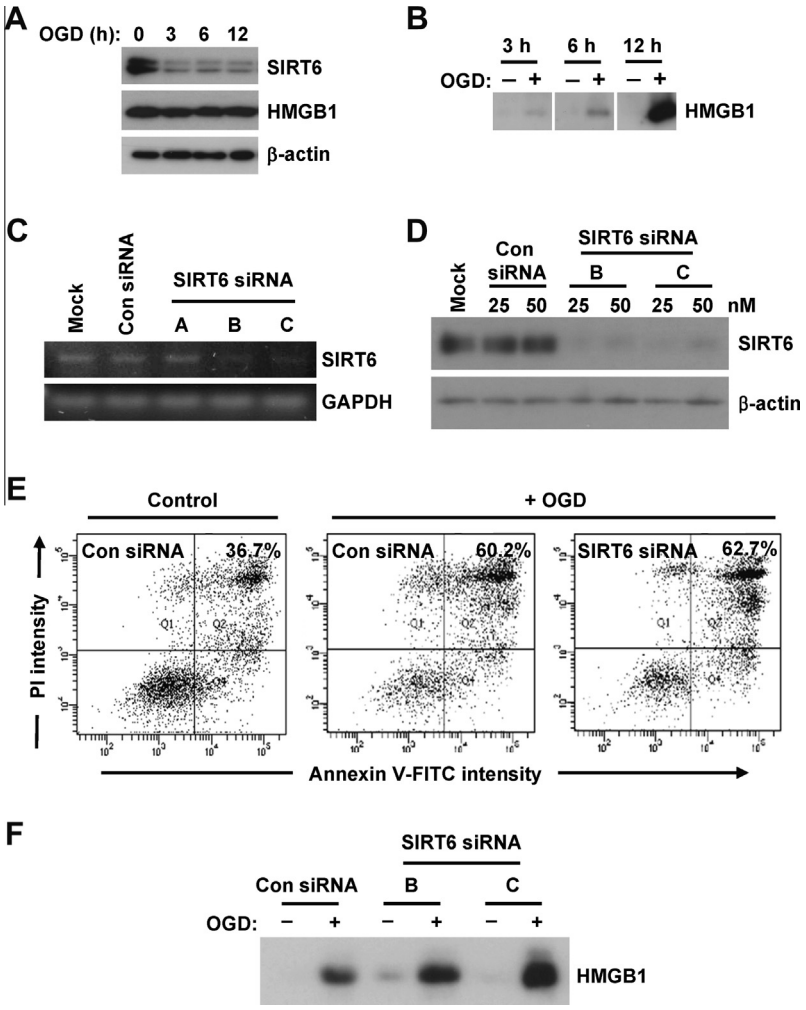

Fig. 4. The downregulation of SIRT6 expression and the release of HMGB1 to the extracellular space in *in vitro* ischemic model. (A) OGD reduces the SIRT6 expression in SH-SY5Y cells. The expression levels of SIRT6 and HMGB1 were analyzed in total cell extracts from SH-SY5Y cells immediately after treatment with OGD for 0, 3, 6, or 12 h. β-actin was used as an internal control. (B) HMGB1 is released from OGD-treated SH-SY5Y cells. SH-SY5Y cells were subjected to OGD for the indicated times. Conditioned medium was analyzed by Western blot using an anti-HMGB1 antibody. (C and D) The knockdown of SIRT6 expression by SIRT6 siRNA. SH-SY5Y cells were transfected with three different SIRT6 siRNAs. The expression of SIRT6 mRNA and protein was analyzed by RT-PCR (C) and Western blot (D), respectively. Con siRNA indicates control siRNA. (E) The effect of SIRT6 knockdown on OGD-induced cell death. SH-SY5Y cells transfected with control or SIRT6 siRNA were cultured under OGD condition for 3 h. Cell death was determined by FACS analysis after staining with Annexin-V and PI. The percentage of cells in late apoptosis or cell death [Annexin-V (+)/PI (+)] are shown. (F) Reduced SIRT6 expression enhances the OGD-induced release of HMGB1. SH-SY5Y cells were treated with 50 nM of SIRT6 siRNA. After 24 h, the cells were exposed to OGD for 3 h. The conditioned medium was analyzed by Western blot analysis using anti-HMGB1 antibody.

model, the inhibition of NF-κB activation also attenuated HMGB1 translocation from the nucleus to the cytoplasm in myoblasts [22]. Recently, SIRT6 was also identified as an inhibitor of NF-κB. SIRT6 interacts with the NF-κB subunit RelA to deacetylate histone H3K9 on NF-κB target gene promoters, while also inhibiting the transcriptional activation of NF-κB [4]. As such, the translocation of HMGB1 by SIRT6 downregulation may also result from the lack of SIRT6 suppression of NF-κB activation.

In this study, we found that neuronal cells represent the primary cell type that highly expresses SIRT6. The levels of SIRT6 in neuron were susceptible to ischemia, and were associated with the release of HMGB1 that participates in brain damage. Controlling the SIRT6 signaling may prove promising in developing new therapeutic interventions targeting ischemic brain damage.

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