Sirtuin 2 (SIRT2) Enhances 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Nigrostriatal Damage via Deacetylating Forkhead Box O3a (Foxo3a) and Activating Bim Protein*

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Background: The functional role of SIRT2 in the MPTP model of Parkinson disease is not known.

Results: Deletion of SIRT2 rescues MPTP-induced nigrostriatal damage by increasing acetylated Foxo3a levels, decreasing Bim expression, thereby preventing apoptotic pathways.

Conclusion: SIRT2 deacetylates Foxo3a, increases Bim expression, and induces nigrostriatal damage.

Significance: SIRT2 deletion is protective against MPTP-induced nigrostriatal damage in the MPTP model of Parkinson disease.

Sirtuins are NAD-dependent deacetylases that were shown to have beneficial effects against age-related diseases (2, 3). SIRT1 was shown to reduce amyloid-β peptide formation in an Alzheimer disease mouse model and suppress α-synuclein aggregation in an A53T α-synuclein mouse model (4, 5). SIRT2 is a strong protein deacetylase that is highly expressed in brain. It was shown to be an abundant neuronal protein that accumulates in the central nervous system of aging mice (6). SIRT2 was shown to co-localize with microtubules and functions as α-tubulin deacetylase (6). SIRT2 protects against Huntington disease (7). In addition, SIRT2 was shown to protect against dopaminergic cell death in an MPP+ model of PD (7). The functional role of SIRT2 in the MPTP model of Parkinson disease (PD),2 the most common movement disorder and the second most common neurodegenerative disease after Alzheimer disease, is characterized primarily by the loss of dopaminergic neurons. MPTP is the most commonly used dopaminergic neurotoxin that replicates most clinical features of PD, producing reliable and reproducible nigrostriatal damage after systemic administration. It is still the only model that displays reproducible neurodegeneration. The chronic MPTP regimen leads to neurodegeneration via apoptosis (1).

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REPORT: Deletion of Mouse SIRT2 Prevents MPTP-induced Damage

sense-mediated decay of the SIRT2 mRNA (10). All mice were housed at controlled temperature (25 °C) and 12:12-h light-dark cycle.

Plasmids—The plasmids expressing mouse SIRT2 (13813) and Bim (8804) were purchased from Addgene. The SIRT2-shRNA (RMM3981-9579810) and Bim-shRNA (RHS4533-NM006538) plasmids were purchased from Open Biosystems.

Immunohistochemistry—Mice were perfused with 4% paraformaldehyde, cryoprotected, sectioned 40 µm thick, and collected at 150-µm intervals. 12 sections per brain were analyzed. VECTASTAIN kit (Vector Laboratories) was used to perform tyrosine hydroxylase (TH) staining according to manufacturer’s directions using TH antibody (Calbiochem). Nissl staining was performed according to the manufacturer’s protocol (IHC World). TH-positive and Nissl-stained neurons in substantia nigra pars compacta (SNpc) were counted by stereology. TH-positive striatal fibers were assessed by optical density.

Western Blotting and Immunoprecipitation—Mouse brains were homogenized in radioimmune precipitation buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate) including Complete protease inhibitor mixture (Roche Applied Science) and centrifuged, and 100 µg of the supernatant was loaded onto 4–15% gradient SDS-PAGE gels and immunoblotted with anti-SIRT2 (Cell Signaling, 2313, concentration 1:1000), Foxo3a (Abcam, ab47409, concentration 1:1000), Bim (Abcam, ab7888, concentration 1:1000), actin (Millipore, MAB1501, concentration 1:5000), and acetylated lysine (Immunostore, 5630, concentration 1:500) antibodies. Western blots were performed with at least two mice per genotype and age, and the representative blots are shown. For Western blotting using cell extracts, cells were harvested and extracted in radioimmune precipitation buffer, as explained above. The immunoprecipitation was performed using Pierce Direct IP kit (Thermo Fisher Scientific) as recommended by the manufacturer’s directions.

RNA Isolation and Analysis—Total RNA from mouse brains or cells was isolated by using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen) with random primers. The cDNA was then subjected to PCR analysis with gene-specific primers in the presence of SYBR Green (Bio-Rad). Relative abundance of mRNA was obtained by normalization to 18 S ribosomal RNA levels. The primers used for Bim qPCR are 5’-CACCATGGCAAGCAACCTTCTGATG-3’ (forward) and 5’-CTCAATGTCATTCTCCACACC-3’ (reverse). The software used to analyze qPCR data is MxPro-Mx3000P v.4.10 Build 389, Schema 85 Stratagene.

Cells and Transfection—SH-SY5Y cells (ATCC) were transfected using Effectene transfection reagent (Qiagen). MPP+ (Sigma) treatment was performed according to manufacturer’s protocol.

Caspase-3 Activity Assay—The Apo-Alert kit (Clontech) was used to measure caspase-3 activity according to manufacturer’s protocol.

Statistical Analysis—The analysis was performed using two-way ANOVA. The type of statistical analysis used is indicated in each figure legend. Significant differences are demonstrated by single symbols (*, #, e) indicating p < 0.01. Error bars in figures represent S.E.

RESULTS AND DISCUSSION

We wished to test whether the deletion of SIRT2 could rescue MPTP-induced nigrostriatal damage by using an SIRT2 KO mouse model. SIRT2 KO animals show no differences in brain development and gross anatomy. Gross histological examination of the brain reveals normal morphology in SIRT2 KO mice (data not shown) (10). MPTP is the most commonly used dopaminergic neurotoxin that produces reliable and reproducible nigrostriatal damage after systemic administration. It is accepted as a pharmacological model of Parkinson disease (1). The chronic MPTP regimen leads to neurodegeneration via apoptosis (1). We thus administered MPTP intraperitoneally to 3-month-old SIRT2 KO mouse and its wild type (wt) littermates via chronic regimen (30 mg kg⁻¹ free base MPTP daily for five consecutive weeks (11, 12)). Nigrostriatal damage caused by the chronic dose of MPTP is the death of dopaminergic neurons (11). The number of dopaminergic neurons and striatal fibers was assessed by TH staining.

Because chronic administration of MPTP induces apoptotic neuronal death in mouse brains, we wanted to analyze whether silenced or overexpressing SIRT2 affects the MPP⁺-induced apoptosis in SH-SY5Y (neuroblastoma) cells. Therefore, we overexpressed or silenced SIRT2 in SH-SY5Y cells and assessed apoptosis. We used caspase-3 activity as a measure of MPP⁺-induced apoptosis. Caspase-3 is an active cell death protease involved in the execution phase of apoptosis, where cells undergo morphological changes such as DNA fragmentation, chromatin condensation, and apoptotic body formation (13). Caspase-3 is activated in response to treatment with pharmacological agents such as MPP⁺. SH-SY5Y cells were treated with media alone or MPP⁺ to a final concentration of 500 µM for different time intervals (2, 4, 6, 8, 12, and 16 h) (14). Caspase-3 activity in cells treated for 16 h with medium alone was comparable with the activity in untreated cells (0 h, Fig.
Deletion of SIRT2 reduces the nigrostriatal damage in mouse brains. A, the left panel shows TH-positive neurons in the SNpc in saline- or MPTP-dosed wt or SIRT2 KO mice. Scale bar represents 50 μm. The quantification on the right shows the number of TH-positive neurons counted by stereology in saline- or MPTP-dosed wt or SIRT2 KO mice. Error bars represent S.E. of three independent experiments. Statistical analyses were carried out using two-way ANOVA. *, p < 0.01, wt saline versus wt MPTP. B, the left panel shows representative Western blot of TH-positive striatal fibers in saline- or MPTP-dosed wt or SIRT2 KO mice. The panel shows the TH-positive striatal fibers in saline-dosed wt mice. Scale bar represents 50 μm. The quantification on the right shows the optical density of the TH-positive fibers in saline- or MPTP-dosed wt or SIRT2 KO mice. Error bars represent S.E. of three independent experiments. Statistical analyses were carried out using two-way ANOVA. *, p < 0.01, wt saline versus wt MPTP. C, the left panel shows representative Western blot of TH-positive striatal fibers in saline- or MPTP-dosed wt or SIRT2 KO mice. The panel shows the TH-positive striatal fibers in saline-dosed wt mice. Scale bar represents 50 μm. The quantification on the right shows the optical density of the TH-positive fibers in saline- or MPTP-dosed wt or SIRT2 KO mice. Error bars represent S.E. of three independent experiments. Statistical analyses were carried out using two-way ANOVA. *, p < 0.01, wt saline versus wt MPTP. D, SIRT2 deacetylates Foxo3a, increases Bim levels, and leads to caspase-3 activity. This result indicates that silencing SIRT2 prevents MPP⁺-induced apoptosis in SH-SY5Y cells.

2A). MPP⁺ treatment increased caspase-3 activity only after 8 h of treatment, reaching the highest level after 16 h (Fig. 2A).

To test the effect of SIRT2 on MPP⁺-induced apoptosis, SH-SY5Y cells were transfected with empty vector (wt), SIRT2 plasmid to overexpress SIRT2, or SIRT2-shRNA plasmid to silence SIRT2 (see “Experimental Procedures”). Fig. 2B shows the SIRT2 levels in cell lysates after transfection. 48 h after transfection, cells were treated with MPP⁺ for 16 h, and then caspase-3 activity was analyzed. SIRT2 overexpression or silencing had no effect on caspase-3 activity in the absence of MPP⁺ treatment (Fig. 2C). After 16 h of MPP⁺ treatment, caspase-3 activity was increased in wt cells (Fig. 2C, vector). SIRT2 silencing decreased caspase-3 activity to baseline, whereas overexpressing SIRT2 increased caspase-3 activity. Scrambled shRNA did not have any effect on caspase-3 activity. This result indicates that silencing SIRT2 prevents MPP⁺-induced apoptosis in SH-SY5Y cells.
SIRT2 was shown to promote cell death when cells are under severe stress by activating Bim, a pro-apoptotic factor (9). It was shown that in cell culture, SIRT2 deacetylates Foxo3a. Because Bim is a pro-apoptotic factor that is one of the target genes of Foxo3a, we analyzed whether SIRT2 deacetylates Foxo3a in SH-SY5Y cells and elevates Bim expression. To measure the acetylation level of Foxo3a in MPP⁺-treated cells where SIRT2 is overexpressed or silenced, we immunoprecipitated Foxo3a from the extracts of cells transfected with control vector, SIRT2 plasmid, or SIRT2 shRNA plasmid using Foxo3a antibody. We then blotted the eluates with acetylated lysine antibody to detect the acetylation of Foxo3a (Fig. 2D). The acetylation levels of Foxo3a were not changed in the absence of MPP⁺ treatment (no MPP⁺) where SIRT2 was silenced or overexpressed (Fig. 2D, left panel). After 16 h MPP⁺ treatment, we observed that the acetylation levels of Foxo3a were decreased in SIRT2-overexpressing cells and increased in SIRT2-silenced cells when compared with empty vector. This result demonstrates that SIRT2 deacetylates Foxo3a in SH-SY5Y cells only after MPP⁺ treatment (Fig. 2D, right panel).

We then tested whether the deacetylation of Foxo3a by SIRT2 elevates the expression level of Bim in cells. We first analyzed the RNA levels of Bim (Fig. 2E). After 16 h of MPP⁺ treatment, Bim RNA levels were increased with overexpressing SIRT2 and decreased with silencing SIRT2. There was no difference in Bim RNA levels with SIRT2 overexpression or silencing Bim. Caspase-3 activity was determined when SIRT2 was overexpressed and decreased when SIRT2 was silenced. Overexpressing Bim increased caspase-3 activity to similar levels as in SIRT2 overexpression. In addition, SIRT2 was silenced. Overexpressing Bim increased caspase-3 activity to similar levels as in SIRT2 overexpression. Importantly, when we overexpressed SIRT2 in MPP⁺-treated Bim-silenced cells, caspase-3 activity was in the levels of wt cells (vector) and not as high as the case of SIRT2 overexpression. This indicates that elevated Bim expression is the main cause of apoptosis. In addition, caspase-3 activity in cells expressing both SIRT2-shRNA and Bim-shRNA was not significantly lower than cells expressing SIRT2-shRNA or Bim-shRNA alone. The latter experiment suggests that SIRT2 and Foxo3a function in the same pathway to elevate caspase-3 activ-
Scrambled shRNAs did not have any effect on the caspase-3 activity (Fig. 3D). Fig. 3E indicates the protein levels of SIRT2 (left panel) or Bim (right panel) after silencing or overexpression in cells of SIRT2 or Bim, respectively. We also analyzed whether the expression level of SIRT2 is up-regulated in MPP⁺⁺-treated cells or MPTP-treated mice by Western blotting. We did not observe any change in SIRT2 protein levels in MPP⁺⁺-treated cells when compared with control cells or in MPTP-injected mice when compared with control mice, indicating that the increase in Bim expression levels is not caused by the increase in SIRT2 expression (Fig. 3F).

These data show that SIRT2 leads to neurodegeneration in MPTP-injected mice and MPP⁺⁺-treated cells by deacetylating Foxo3a, increasing Bim levels, and therefore leading to apoptosis. We also show that deletion of SIRT2 prevents neuronal death in MPTP-treated mice. Similarly, silencing SIRT2 in MPP⁺⁺-treated cells also inhibits apoptosis. The results shown here are also consistent with the fact that sirtuins are stress-response genes. In this study, SIRT2 is shown to deacetylate Foxo3a, increasing Bim levels, and therefore leading to apoptosis. We did not observe any change in SIRT2 protein levels in Parkinson disease mouse model (A53T). SIRT1 was shown to deacetylate HSF1 and increase Hsp70 levels only after heat shock in cells or only in the brains of a stress condition and turn on their target pathways. Therefore, studies show that SIRT1 and SIRT2 are activated as a result of the disease condition might be useful in developing treatments for Parkinson disease.

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In Figs. 2D, 3A, and 3E, the samples were run on gels with 18 wells, which contain repetition of some lanes and also combination of different experiments. The samples of interest were run on different parts of the same gel. To make it easy for the readers to interpret, the samples of interest were spliced together. However, the splicing was not made clear by insertion of dividing lines. Corrected versions of the figures are shown below with the insertion of dividing lines between the spliced lanes.

In addition, in Figs. 2D and 3A, the labels “NRS” and “SIRT2” on the top are supposed to show the NRS or the antibody that was used to coat the beads in the immunoprecipitation assay, respectively. The beads were coated with NRS or Foxo3a antibody. “Foxo3a” should have been used as a label and not “SIRT2.” This error has been corrected.

Due to an error during the preparation of Fig. 3F (right panel), a “no MPTP” sample was placed instead of an “MPTP” sample. This is now corrected in the figure shown below. The replacement figure is the original gel.

The corrected images in no way affect the conclusions of the paper or the original interpretation of the results. The authors apologize for any confusion caused by this error.