NAMPT protects against 6-hydroxydopamine-induced neurotoxicity in PC12 cells through modulating SIRT1 activity

XIAO-DONG ZOU[1*], SHAO-QING GUO[2*], ZHI-WEI HU[1] and WEI-LANG LI[1]

[1]Department of Neurology, Tongde Hospital of Zhejiang Province; [2]Department of Massage, The Third Affiliated Hospital of Zhejiang Province Traditional Chinese Medical University, Hangzhou, Zhejiang 310012, P.R. China

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Abstract. Parkinson's disease (PD) is the second most common progressive neurodegenerative movement disorder. Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the first rate-limiting step in the nicotinamide adenine dinucleotide (NAD^+) biosynthetic pathway in mammals, is a substrate for NAD^+-dependent enzymes, such as sirtuin 1 (SIRT1), and contributes to cell fate decisions. However, the role of NAMPT in PD has remained to be fully elucidated. In the present study, PC12 cells were treated with the neurotoxin 6-hydroxydopamine (6-OHDA) to establish an *in vitro* model of PD, following which an obvious inhibitory effect on the levels of NAMPT and NAD^+ as well as the NAD^+/NADH ratio was detected. In addition, pre-incubation with FK866, a highly specific NAMPT inhibitor, enhanced the inhibitory effects of 6-OHDA on the viability of PC12, while pre-incubation with nicotinamide mononucleotide (NMN), am enzymatic product of NAMPT, had the opposite effect. Furthermore, it was revealed that NMN markedly attenuated 6-OHDA-induced decreases in superoxide dismutase activity and glutathione levels, as well as 6-OHDA-induced increases in malondialdehyde and lactate dehydrogenase in PC12 cells. Furthermore, 6-OHDA significantly reduced SIRT1 activity in PC12 cells, which was inhibited by NMN. The pharmacological activator resveratrol also significantly inhibited 6-OHDA-mediated decreases in PC12 cell viability while reversing 6-OHDA-induced decreases in SIRT1 levels. The results of the present study suggested that NMT protected against 6-OHDA-induced decreases in PC12 cell viability, and that SIRT1 activation had a role in this process. Treatment with NMN to activate SIRT1 may represent a novel therapeutic strategy for treating PD.

Correspondence to: Dr Xiao-Dong Zou, Department of Neurology, Tongde Hospital of Zhejiang Province, 234 Gucui Road, Xihu, Hangzhou, Zhejiang 310012, P.R. China
E-mail: zou_xiaodong12@163.com

*Contributed equally

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Introduction

Parkinson's disease (PD), a chronic and progressive neurodegenerative movement disorder, is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) (1). The mechanisms of dopaminergic cell death have remained to be fully elucidated. Lipid peroxidation, oxidative stress and mitochondrial dysfunction are considered to mediate the progression of dopaminergic neuron degeneration (2). As the etiology and pathogenesis of PD have remained elusive, current therapies focus on the symptoms, rather than the neurodegenerative progression of dopaminergic neurons in SNc. Therefore, novel insight into the disease mechanisms is urgently required. 6-Hydroxydopamine (6-OHDA), which has been previously used to establish *in vivo* as well as *in vitro* models of PD, is a neurotoxin mainly targeting dopaminergic neurons (3,4). 6-OHDA exerts its effects via inducing reactive oxygen species (ROS) overproduction and energy depletion (1,5). PC12 cells are widely used for *in vitro* studies exploring the mechanisms of neurodegenerative diseases (6-8).

Seven members of the sirtuin family have been associated with energy production, cell metabolism and DNA repair in mammals. They are deacetylases acting on histones in the presence of nicotinamide adenine dinucleotide (NAD^+) (9). Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in the mammalian NAD^+ salvage pathway for the conversion of nicotinamide into nicotinamide mononucleotide (NMN), which is later converted to NAD^+ (10). NAMPT is thought to function in a manner equivalent to that of pyrazinamidase/nicotinamidase 1 in mammals (11), and increased expression of NAMPT has been shown to positively regulate NAD^+ levels and enhance SIRT1 transcriptional regulatory activity in mouse fibroblasts (12). The NAD^+/NADH ratio is a fundamental indicator of the cellular redox status (13) and ROS accumulation has been shown to modify the activity of sirtuins (14). Furthermore, a previous study demonstrated that NMN protected the rotenone-induced PC12 cells from cell death by restoring the intracellular levels of NAD^+ and preventing ATP depletion (15).

SIRT1 has beneficial effects on numerous major aging-associated pathologies, including diabetes (16), neurodegeneration (17), chronic heart failure (18) and cancer (19). Resveratrol treatment was shown to attenuate cell stress caused by to caloric restriction via activation of SIRT1 (20). SIRT1 also regulates an array of transcription factors, including nuclear factor κB (21) and p53 (22). High levels of ROS induce the expression of SIRT1 (23), which in turn initiates DNA repair (24).

In the present study, the neuroprotective effects of the NAMPT metabolite NAD^+ against 6-OHDA-induced neurotoxicity in PC12 cells were elucidated. Furthermore, the possible underlying mechanisms were examined by using an NAMPT inhibitor and by assessing effects on indicators of oxidative damage as well as SIRT1 activation.

Materials and methods

Cell culture and treatment. The PC12 rat adrenal pheochromocytoma cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% heat-inactivated equine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). Cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2. The medium was replaced every two or three days and cells were routinely subcultured at a 1:5 ratio at weekly intervals. For the experiments, PC12 cells were pre-incubated with NMN (600 μM), FK866 (10 nm) or resveratrol (50 μM; Sigma-Aldrich) for 2 h and then exposed to 6-OHDA for 24 h. The control group was treated with an equivalent volume of dimethyl sulfoxide (DMSO; final concentration, 0.1%; Sigma-Aldrich) added to the medium.

Cell viability assay. Cell viability was estimated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, PC12 cells were seeded in 96-well plates at 10,000 cells/well for 24 h. After 6-OHDA treatment for 24 h with or without pre-incubation with NMN, FK866 or resveratrol, the cells were incubated with 20 μl MTT solution (5 mg/ml; Sigma-Aldrich) for 4 h. The culture medium was removed and the dark blue formazan product was dissolved by adding 150 μl DMSO to each well. The absorbance of each well was read at 570 nm using the Rayto-RT 6000 enzyme-linked immunosorbent assay reader (Rayto Life and Analytical Sciences, Guangdong, China), and the viability was expressed as the percentage of the untreated cells.

NAD^+/NADH and SIRT1 activity assays. An NAD^+/NADH quantification kit was used to determine NAD^+ and NADH levels (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. To assess SIRT1 activity, whole-cell extracts were prepared using a mild lysis buffer plus protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). An Infinite M200 microplate fluorimeter (Tecan, Hillsborough, NC, USA) was used to measure SIRT1 activity.

Assessment of glutathione (GSH) and malondialdehyde (MDA) levels. PC12 cells were washed with 1 ml phosphate-buffered saline (PBS) three times and subsequently scraped off the bottom of the flask in ice-cold PBS. Lysis buffer was then added to the cells, followed by incubation for 40 min on ice. The lysates were centrifuged at 10,000 x g for 10 min at 4˚C. The supernatant was collected and the protein content was determined using the bicinchoninic acid (BCA) protein assay kit, followed by analysis of the GSH and MDA content using a commercial colorimetric GSH and MDA assay kit (Beyotime Institute of Biotechnology, Haimen, China).

Assessment of superoxide dismutase (SOD) activity and lactate dehydrogenase (LDH). According to the manufacturer's instructions, 100 μl culture supernatant was used to determine SOD activity using a commercially available detection kit (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China). For the LDH assay, 80 μl culture supernatant was collected and subjected to analysis using an LDH assay kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China) according to the manufacturer's instructions.

Western blot analysis. Western blot analysis was performed according to the protocol of a previous study (25). PC12 cells (10^6 cells/well) were seeded onto six-well plates and treated with 6-OHDA for 24 h with or without pre-incubation with FK866, NMN or resveratrol. Subsequently, cells were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing protease inhibitor cocktail (Roche Diagnostics) and then incubated on ice for 30 min. The cellular debris was removed by centrifugation (14,000 x g for 10 min at 4˚C) and the protein concentration in the supernatant was determined using a BCA protein assay kit. Equal amounts of protein (20-30 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking in 5% non-fat milk for 1 h at room temperature, blots were incubated with rabbit monoclonal anti-NAMPT (1:1,000; Abcam, Cambridge, MA, USA; cat. nos. ab45890), anti-SIRT1 antibody (1:1,000; cat. no. ab32441) and mouse monoclonal β-actin (1:1,000; cat. no. ab6276) overnight at 4˚C. Subsequent to three washes in Tris-buffered saline containing Tween 20, membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG or goat anti-mouse horseradish peroxidase-conjugated IgG (1:5,000; Beyotime Institute of Biotechnology; cat. nos. A0208 and A0216, respectively) for 1 h at room temperature. Proteins were detected using the enhanced chemiluminescence western blot detection kit (Amersham ECL plus Western blotting detection system; GE Healthcare). The blots were washed again and scanned. The proteins of interest were detected using the Odyssey Western Detection system and quantified with the Odyssey LI-COR System software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. Experiments were repeated at least three times with consistent results. Values are expressed as the mean ± standard error of the mean and one-way analysis of variance followed by Tukey's multiple comparisons test was

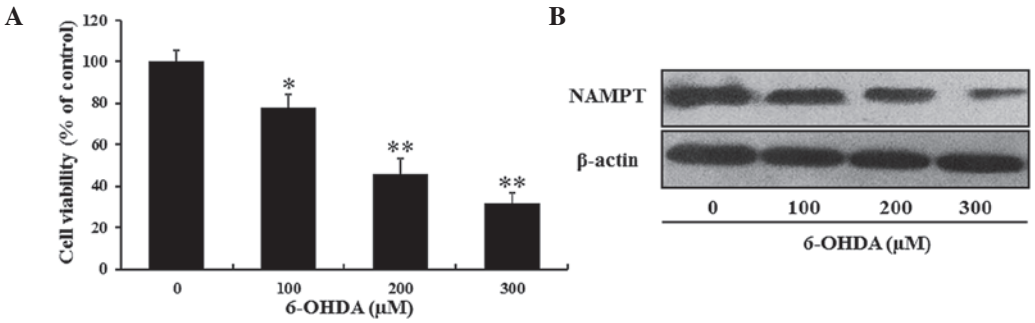

Figure 1. Effects of 6-OHDA treatment on the viability of PC12 cells and expression of NAMPT. PC12 cells were incubated with 6-OHDA (0, 100, 200 and 300 μM) for 24 h. (A) MTT assay revealed that 6-OHDA dose-dependently decreased cell viability. Values are expressed as the mean ± standard error (n=3). *P<0.05 and **P<0.01 vs. the control group. (B) Western blot analysis revealed that the expression of NAMPT in PC12 cells is downregulated by 6-OHDA in a dose-dependent manner. NAMPT, nicotinamide phosphoribosyltransferase; 6-OHDA, 6-hydroxydopamine.

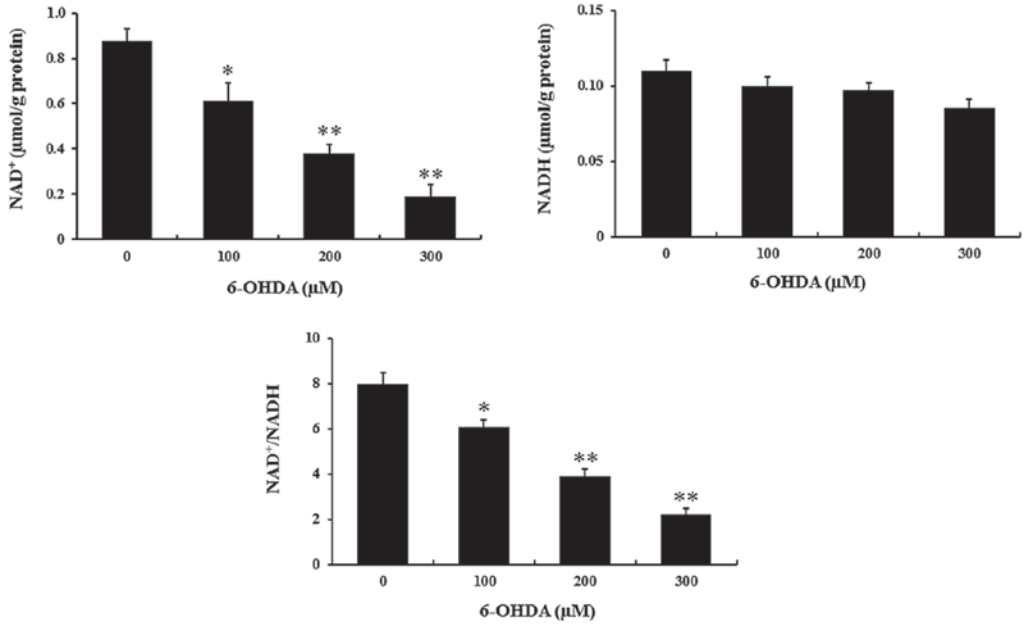

Figure 2. 6-OHDA dose-dependently decreases the levels of NAD^+ and the NAD^+/NADH ratio in PC12 following treatment for 24 h. Values are expressed as the mean ± standard error of the mean (n=4). *P<0.05 and **P<0.01 vs. the control group. 6-OHDA, 6-hydroxydopamine; NAD, nicotinamide adenine dinucleotide.

used for comparisons between multiple groups. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

6-OHDA-induced decreases in PC12 cell viability are accompanied with a reduction in NAMPT and NAD^+ levels. According to the protocols of previous studies (8,26), the present study generated a 6-OHDA-induced *in vitro* model of neurodegeneration resembling PD. PC12 cells were treated with various concentrations of 6-OHDA for 24 h and the cell viability was determined using an MTT assay. In accordance with the findings of the previous studies, the viability of PC12 cells was decreased by 6-OHDA in a dose-dependent manner. Only ~50% of PC12 cells survived after exposure to 200 μM 6-OHDA for 24 h (Fig. 1A).

To investigate the effects of 6-OHDA-induced neuronal cell death on NAMPT levels in PC12 cells, western blot analysis was performed. Compared with the control group, NAMPT expression was markedly decreased by 6-OHDA in a dose-dependent manner (Fig. 1B).

As studies have indicated that NAD^+ levels are regulated by NAMPT in mammalian neurons, the present study detected the concentration of NAD^+ and NADH in response to 6-OHDA treatment. Compared with that in the control group, the NAD^+ release was significantly decreased by 6-OHDA treatment (P<0.01) (Fig. 2), while NADH levels were not affected. Consequently, the NAD^+/NADH ratio was reduced by 6-OHDA in a dose-dependent manner (P<0.01) (Fig. 2).

NMN protects PC12 cells against 6-OHDA-induced death, while NAMPT inhibition aggravates 6-OHDA-induced PC12 cell death. In order to determine the roles of NAMPT in the molecular pathology of PD, PC12 cells were

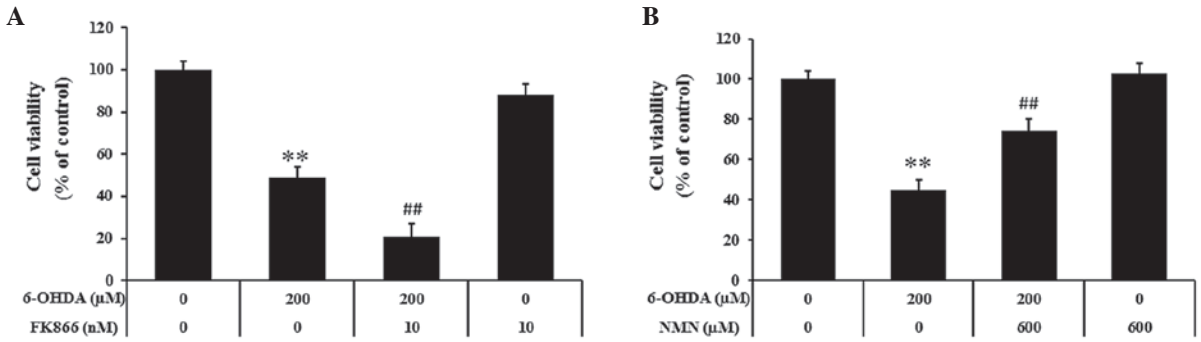

Figure 3. FK866 enhances, while NMN attenuates the inhibitory effects of 6-OHDA on PC12 cell viablilty. (A) PC12 cells were pre-treated with nicotinamide phosphoribosyltransferase inhibitor FK866 for 1 h, followed by incubation with 200 μM 6-OHDA for 24 h. (B) PC12 cells were incubated with 200 μM 6-OHDA for 24 h after pre-treatment with NMN for 1 h. Values are expressed as the mean ± standard error (n=3). **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. 6-OHDA group. 6-OHDA, 6-hydroxydopamine; NMN, nicotinamide mononucleotide.

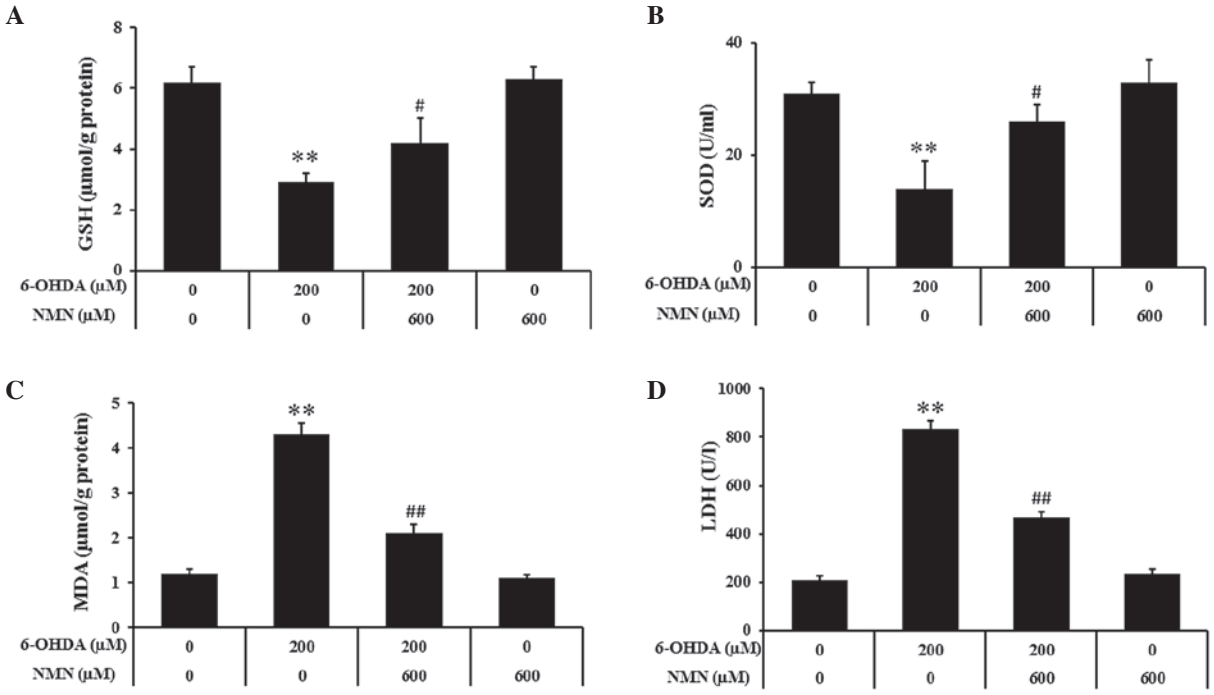

Figure 4. Pre-treatment with NMN prevents 6-OHDA-induced oxidative damage and LDH release by PC12 cells. Effects of 6-OHDA and/or NMN on (A) GSH, (B) SOD, (C) MDA and (D) LDH levels in PC12 cells after treatment with 6-OHDA for 24 h. Values are expressed as the mean ± standard error of the mean (n=3). **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. 6-OHDA group. SOD, superoxide dismutase; LDH, lactate dehydrogenase; GSH, glutathione; NMN, nicotinamide mononucleotide; MDA, malondialdehyde; 6-OHDA, 6-hydroxydopamine.

pre-treated with FK866, a NAMPT-specific inhibitor, or NMN, the enzymatic product of NAMPT, prior to induction with 6-OHDA. As shown in Fig. 3A, pre-incubation with FK866 (10 nM) significantly decreased the viability of PC12 cells following treatment with 6-OHDA from ~50 to 21% (P<0.01). Furthermore, PC12 cells were pre-incubated with NMN (600 μM) followed by incubation with 6-OHDA (200 μM) for 24 h (Fig. 3B). It was observed that NMN markedly increased the viability of 6-OHDA-treated PC12 cells from ~45 to 74% (P<0.01). These results demonstrated that following induction of PC12 cells with 6-OHDA, prior inhibition of NAMPT activity decreased the cell viability, while NMN, the enzymatic product of NAMPT, rescued cell viability.

NMN prevents 6-OHDA-induced oxidative damage of PC12 cells and LDH release. Previous studies have demonstrated that oxidative stress is as an important mediator in 6-OHDA-induced cell death (27,28). To explore whether NMN protects PC12 cells against 6-OHDA induced cell death due to its anti-oxidant capacity, the levels of GSH and SOD, which are the most common indicators of anti-oxidant activity, were assessed in PC12 cells. As a vital enzymatic anti-oxidant, SOD has an important role in the clearance of ROS. As shown in Fig 4A and B, treatment with 200 μM 6-OHDA significantly reduced the intracellular concentration of GSH and SOD, which was partially inhibited by treatment with 600 μM NMN (P<0.05). Furthermore, the concentration of MDA, a lipid oxidation biomarker, was assessed. After incubation

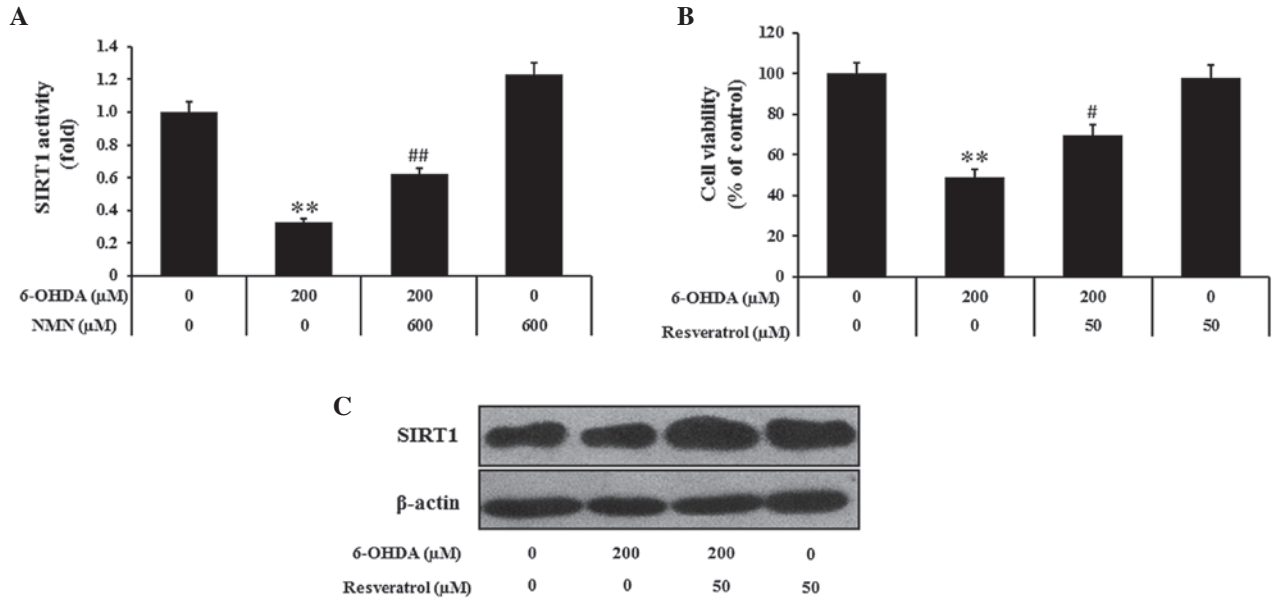

Figure 5. NMN and resveratrol may attenuate 6-OHDA-induced decreases in cell viability via enhancing SIRT1 activity. (A) Pre-treatment with NMN enhances SIRT1 activity in PC12 cells after treatment with 6-OHDA for 24 h. (B) Resveratrol reduces the inhibitory effects of 6-OHDA on PC12 cell viability. Values are expressed as the mean ± standard error of the mean (n=3). **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the 6-OHDA group. (C) Pre-treatment with resveratrol enhances SIRT1 activity in PC12 cells after treatment with 6-OHDA for 24 h. SIRT, sirtuin; NMN, nicotinamide mononucleotide; 6-OHDA, 6-hydroxydopamine.

with 6-OHDA for 24 h, the levels of MDA were significantly increased (P<0.01), which was attenuated by NMN (600 μM) (P<0.01) (Fig. 4C). The release of LDH into the culture medium due to plasma membrane damage is an indicator of cell death. As shown in Fig. 4D, 6-OHDA markedly enhanced LDH release, which was significantly attenuated by NMN (P<0.01). All of these results indicated that NMN protects PC12 cells from 6-OHDA-induced death due to its anti-oxidant activity.

NMN may exert its neuroprotective effects by activating SIRT1. Since SIRT1 is known to mediate several biological effects of NAMPT, the present study further assessed whether the protective effects of NMN against 6-OHDA-induced PC12 cell death may be mediated via modulation of SIRT1 activity. Indeed, the results indicated that 6-OHDA markedly decreased the activity of SIRT1 compared to that in the control group, while NMN attenuated this effect (P<0.01) (Fig. 5A). The pharmacological activator resveratrol is known to exert protective effects on PC12 cells against 6-OHDA-induced toxicity. Pre-treatment with resveratrol (50 μM) markedly reduced the inhibitory effects of 6-OHDA on cell viability (P<0.05) (Fig. 5B). Furthermore, western blot analysis showed that the 6-OHDA-induced reduction of SIRT1 expression was reversed by pre-treatment with resveratrol (Fig. 5C). All of these results indicated that enhanced SIRT1 activity and increased SIRT1 expression are associated with the protective effects of NMN against 6-OHDA-induced PC12 cell death.

Discussion

The present study demonstrated that NMN, the enzymatic product of NAMPT, exerts protective effects against 6-OHDA-induced neurodegeneration *in vitro*. It was revealed that following 6-OHDA treatment, PC12 cell viability was decreased, NAMPT was downregulated, and the levels of NAD^+ as well as the NAD^+/NADH ratio were significantly decreased. Indicators of cell death and oxidative damage were increased, and SIRT1 activation was decreased following treatment with 6-OHDA, which was attenuated by treatment with NMN. Furthermore, the NAMPT inhibitor FK866 was shown to aggravate the cytotoxic effects of 6-OHDA. The present study therefore indicated that decreases in NAMPT/NAD+ and subsequent deactivation of SIRT1 may be implicated in the molecular pathology of neurodegenerative disorders, including PD.

The neurotoxin 6-OHDA induces dopaminergic neuronal death and has been widely used to establish *in vitro* models of PD (1). 6-OHDA exerts its toxic effects via inducing ROS overproduction, which results in oxidative stress and cell death, through the following pathways: Extracellular auto-oxidation, monoamine oxidase-mediated intracellular metabolism and mitochondrial respiratory chain inhibition (29,30). PC12 cells have been widely used in studies examining the molecular mechanisms of neurodegenerative diseases (6,8). Therefore, 6-OHDA-treated PC12 cells were used in the present study as a cell model to investigate the implication of NAMPT in PD.

In the present study, 6-OHDA was shown to concentration-dependently reduce NAD^+ levels; this may have been a consequence of the simultaneously observed reduction of NAMPT, as NAMPT is required for the production of NAD^+ (31). While NADH levels were not affected by 6-OHDA, the NAD^+/NADH ratio was decreased, which may explain for the observed reduced cell survival rate due to various stressors (32). In addition, increases in NAMPT or NMN have been previously indicated to markedly reduce cell death (31), which was consistent with the observations of the present study that NMN enhanced the survival of PC12 cells after incubation with 6-OHDA. Furthermore, pre-incubation

with the NAMPT inhibitor FK866 aggravated the cytotoxic effects of 6-OHDA, which further suggested the neuroprotective effects of NAMPT/NMN.

In neurodegenerative disorders, oxidative stress originates from ROS overproduction and impaired anti-oxidative defense is a major reason of neuronal death (33). The antioxidant defense system consists of non-enzymatic anti-oxidants such as GSH and enzymes such as SOD, which neutralize free radicals. The present study revealed that, compared with those in the control group, the levels of GSH and SOD were markedly reduced in 6-OHDA-treated PC12 cells, which was attenuated by pre-treatment with NMN. These results suggested that NMN, an enzymatic product of NAMPT, attenuated the 6-OHDA-induced increases in the levels of anti-oxidants. It is therefore indicated that NAMPT may have an anti-oxidant role in PC12 cells and may reduce the consumption of other anti-oxidants, including GSH and SOD. Furthermore, the levels of MDA, a biomarker of lipid oxidation, were markedly increased after 6-OHDA treatment further confirming that 6-OHDA exerts its neurotoxic effects by inducing oxidative stress in PC12 cells. However, pre-treatment with NMN significantly reduced MDA levels in PC12 cells following 6-OHDA treatment, further indicating its anti-oxidant effects. In brief, NAMPT could enhance the ability of scavenging anti-oxidant action and the free radicals in by 6-OHDA induced PD cell models.

SIRT1 is a unique protein deacetylator and is closely linked to cellular survival due to its dependence on NAD^+ (34). In the present study, it was demonstrated that 6-OHDA decreased SIRT1 activation in PC12 cells, which was attenuated by NMN. Furthermore, the neuroprotective compound resveratrol inhibited 6-OHDA-induced toxicity and increased the expression of SIRT1 in PC12 cells. These results indicated that NAMPT/NMN may exert their neuroprotective/anti-oxidant effects via activation of SIRT1.

In conclusion, the present study demonstrated that NAMPT, a rate-limiting enzyme for mammalian NAD^+, markedly protected PC12 cells against 6-OHDA-induced oxidative stress-associated cell death. The protective effects of NAMPT/NMN may be attributed to, at least in part, their potent anti-oxidant properties, as evidenced by the marked increases in GSH and SOD as well as the reduction of MDA, through enhancing SIRT1 activity. NAMPT and NAD as well as SIRT1 may therefore have a crucial role in PD and other neurodegenerative disorders, and their upregulation may represent a novel therapeutic strategy.

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