HDAC inhibitor trichostatin A-inhibited survival of dopaminergic neuronal cells

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A R T I C L E   I N F O

Article history:
Received 21 July 2009
Received in revised form 30 September 2009
Accepted 9 October 2009

Keywords:
Dopaminergic neuron
HDAC inhibitors
Parkinson’s disease
Neurotoxicity

A B S T R A C T

Histone deacetylase (HDAC) inhibitors have been shown associated with neurodegenerative diseases. However, their effects on survival of dopaminergic neurons remain uncertain. In the present study, the HDAC inhibitor trichostatin A (TSA) was tested in following dopaminergic neuronal cell lines: rat N27, mouse MN9D, and human SH-SY5Y cells. Results demonstrated that a single TSA treatment resulted in decreased cell survival and increased apoptosis in dopaminergic neuronal cells. Pre-treatment with TSA resulted in exacerbated neurotoxic damage to dopaminergic neurons induced by 1-methyl-4-phenylpyridinium and rotenone. These results suggest that HDAC inhibitors may influence Parkinson’s disease pathogenesis by inhibiting survival and increasing vulnerability of dopaminergic neurons to neurotoxins. Our data also suggested the importance of prudent use of HDAC inhibitors in therapy.

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Parkinson’s disease (PD) is a progressive neurodegenerative disease related to aging, and is due to the degeneration and death of dopaminergic neurons in the substantia nigra pars compacta (SNc) of the ventral midbrain [6]. A variety of evidence has suggested that apoptosis may contribute to neuronal death in neurodegenerative disorders, including PD [21,26]. Several neurotoxins, such as 1-methyl-4-phenylpyridinium (MPP+) and rotenone, have been shown to specifically damage dopaminergic neurons [17,21].

Aberrant histone acetylation has been linked to a number of age-related disorders [9,18]. Normally, histone acetylation is one of the major epigenetic processes that control gene transcription [9,18]. Histone deacetylase (HDAC) inhibitors have been shown to lead to histone acetylation and activate gene transcription [8,18]. Moreover, histone acetylation and HDAC inhibitors may be involved in the control of apoptosis, cell survival, differentiation, energy metabolism, and response to internal and external environmental factors [9,13,22]. Recent investigations also suggest that HDAC inhibitors might be associated with neurodegenerative diseases [5,11,16]. Chen et al. found that HDAC inhibitors could induce microglial apoptosis and attenuate lipopolysaccharide-induced dopaminergic neurotoxicity in inflammation-related neurodegenerative disorders, such as PD [4,5]. Leng et al. found that valproate (VPA), a HDAC inhibitor, could induce endogenous alpha-synuclein and suggested it might be suitable in the treatment of excitotoxicity-related neurodegenerative diseases [16]. However, in some clinic reports, it has been suggested that there might be a cause-and-effect relationship between exposure to VPA and the occurrence of Parkinsonism [2,20,24].

The effects of HDAC inhibitors on PD remain unclear. Importantly, it is very necessary to define effects of HDAC inhibitors on survival of dopaminergic neurons, which are the most important targets in PD pathogenesis and therapy. Moreover, since HDAC inhibition has been suggested to be a therapeutic approach in the treatment of a range of human diseases [4,5,11,16], the pharmacological effects of HDAC inhibition on nerve system require further understanding. To address these questions, we employed HDAC inhibitor trichostatin A (TSA) to treat cultured dopaminergic neuronal cells and investigated neuronal cell survival, apoptosis and neurotoxic cell damage.

The dopaminergic neuronal cell lines, human SH-SY5Y, rat N27, and mouse MN9D, were cultured in RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), and incubated at 37 °C in a humidified chamber with 5% CO2. The neurotoxin MPP+ (Sigma, St. Louis, MO) was dissolved in PBS. The HDAC inhibitor TSA (Sigma, St. Louis, MO), and neurotoxin rotenone (Sigma, St. Louis, MO), were dissolved in DMSO.

Cells were treated at the doses indicated in culture medium containing MPP+ or rotenone, with or without TSA pre-treatment, for the time periods indicated. Treatment with DMSO or PBS was used as the control.

The treated cells were harvested and immediately stained with 0.02% trypan blue (Life Technologies, Inc., Gaithersburg, MD). Cells were then quantified using a hemocytometer under a microscope. Stained black cells were considered to be dead cells, and unstained bright cells were viable cells. Experiments were performed in triplicate.

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doi:10.1016/j.neulet.2009.10.037
Cells were seeded 1 day prior to treatment. All suspended and adherent cells were harvested and washed twice with PBS. The cells were then resuspended in 200 μL binding buffer (PBS containing 1 mM calcium chloride). An annexin V–FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) was used to detect apoptotic activity according to the manufacturer’s instructions. FITC-conjugated annexin V (0.5 mg/mL, final concentration) was added and incubate for 20 min at room temperature. Then binding buffer (400 μL) was added and samples were immediately analyzed with a fluorescent microscope, excitation of 488 nm (Olympus, Japan). Apoptotic cells (annexin V–FITC positive) were microscopically quantified in at least 5 random fields. Experiments were performed in triplicate.

Apoptosis was also judged by DNA fragmentation assays (DNA laddering). Cells were washed twice with PBS and resuspended in 100 μL of TE buffer. Cells were lysed with 100 μL lysis buffer and 20 μL RNaseA/protease K. Following centrifugation, the lystate supernatant was loaded onto binding column (BioDev, Beijing, China) and eluted with 40 μL elution buffer. Genomic DNA was separated on a 1.5% agarose gel. The DNA ladder pattern was observed under ultraviolet light subsequent to ethidium bromide staining (0.5 g/mL).

Protein extraction and Western blot analysis were modified as described in Ref [27]. Cells were suspended in 200 μL lysis buffer (1 M Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 0.1% Triton X-100; 1 mM sodium orthovanadate, 0.1 mM sodium pyrophosphate, 2 μg/mL leupeptin, and 2 μg/mL aprotinin). The cells were disrupted by sonication, and total protein was extracted by centrifuging the tubes at 4 °C for 30 min at maximal speed to remove debris. Total protein (50 μg) was separated on SDS-PAGE, electrotransferred to nitrocellulose membranes, and probed with primary antibodies specific to p53 (1:2000, Cat# 2524, CST, Danvers, MA) or actin (1:2000, Cat# A4700, Sigma, St. Louis, MO). The signal was detected using anti-mouse IDY800 secondary antibody (1:5000, Cat# 610-132-121, Rockland Immunochemicals, Gilbertsville, PA) and scanned with Odyssey® Imaging System (LI-COR Bioscience Inc., USA).

Assays of cell viability and apoptosis were performed in triplicate. Values were presented as mean± SEM. Data analysis was performed using one-way ANOVA and a p value <0.05 was considered statistically significant.

To investigate the effects of HDAC inhibitor on dopaminergic neurons, human SH-SY5Y, rat N27, and mouse MN9D dopaminergic cells were treated with TSA. TSA is a commonly used inhibitor of histone deacetylase [3] and can restore histone acetylation. The present results demonstrated that treatment with high doses of TSA (100, 300, or 600 nM) for 24 h in N27 cells (Fig. 1A and Supplementary Fig. 1), and 48 h in MN9D cells (Fig. 1B), resulted in markedly decreased cell viability, compared with control cells. At lower doses (2–10 or 10–50 nM), for 12–96 h, TSA resulted in decreased numbers of viable cells in a time- and dose-dependent manner in N27 and SH-SY5Y cells (Fig. 1C and D), compared with control cells. Within 12–24 h, TSA treatment of N27 (2, 5, or 10 nM) and SH-SY5Y (10, 25, or 50 nM) cells resulted in little to no cytotoxic effects (Fig. 1C and D).

To determine whether apoptosis accounts for the TSA-induced decreased survival in dopaminergic neuronal cells, the effects of phosphatidyserine exposure to cell membranes was analyzed with annexin V–FITC staining, and also DNA strand breaks were analyzed with the DNA ladder assay. In N27 cells, 12–24 h TSA treatment (300 nM) resulted in increased apoptosis, as detected by annexin V–FITC staining (Fig. 2A and Supplementary Fig. 2). In addition, the DNA ladder assay demonstrated that 8 h TSA treatment (150 nM) resulted in typical apoptosis, as indicated by DNA strand breaks (Fig. 2B). In SH-SY5Y cells, 24 h TSA treatment (300 nM) resulted in less apoptosis, as indicated by weaker DNA strand breaks (Fig. 2B). These results suggested that apoptosis might account for decreased survival in cultured dopaminergic neuronal cells due to TSA treatment.

Fig. 1. Decreased survival of dopaminergic neuronal cells (N27, SH-SY5Y, or MN9D) following TSA treatment. Cell viability assay was performed by trypan blue staining. (A) N27 cells were treated with 100, 300, or 600 nM TSA for 24 h. (B) MN9D cells were treated with 100, 300 or 600 nM TSA for 48 h. (C) The cell growth curve of N27 cells treated with 2, 5, or 10 nM TSA for 12–96 h. (D) The cell growth curve of SH-SY5Y cells treated with 10, 25, or 50 nM TSA for 12–96 h. These values represent mean± SEM of triplicate experiments (n = 3). Columns, mean; bars, SEM.
Production of p53 may be involved in programmed neuronal death [21]. To investigate whether p53 protein might function in TSA-induced apoptosis, we examined p53 protein expression by Western blot. Results demonstrated that TSA (150 nM, 2–8 h) did not alter p53 protein expression (Fig. 2C). These suggested that p53 protein expression might not be influenced in TSA-induced apoptosis of N27 cells.

In PD models, the neurotoxins MPP\(^+\) and rotenone are commonly used to specifically injure dopaminergic neurons. Several HDAC inhibitors have been reported to associate with inflammation-related PD by inducing microglial apoptosis [5]. However, the effects of HDAC inhibitors on neurotoxic damage to dopaminergic neurons remain unclear. Therefore, we pre-treated dopaminergic neuronal cells with low cytotoxic doses of TSA, followed by treatment with MPP\(^+\) or rotenone. Results demonstrated that, in N27 cells, 12 h of TSA pre-treatment (10 nM) exacerbated the neurotoxic damage induced by MPP\(^+\) (200 μM, 12 h) or rotenone (0.5 μM, 12 h) (Fig. 3A and B and Supplementary Fig. 3A and B). Similarly, TSA pre-treatment (50 nM) for 24 h increased the neurotoxic damage due to MPP\(^+\) (400 μM, 24 h) or rotenone (1 μM, 12 h) in SH-SY5Y cells (Fig. 3C and D). These results suggested that TSA might increase the vulnerability of dopaminergic neurons to neurotoxic damage.

Although HDAC inhibitors have been used as epigenetic drugs in several phase I and II clinical trials of human diseases [8,18,19], recent findings indicate that HDAC inhibitors may act on PD in a complex manner. Several HDAC inhibitors have been shown to induce microglial apoptosis and attenuate lipopolysaccharide-induced dopaminergic neurotoxicity [5]. The HDAC inhibitor VPA also stimulates the astrocytic release of neurotrophic factors to protect dopaminergic neurons in midbrain neuronal/glial cultures [4]. On the other hand, there was a significantly higher prevalence of patients with Parkinsonism in the group of patients treated with VPA [12]. In addition, many HDAC inhibitors have been shown to inhibit cell proliferation and stimulate neuronal apoptosis [7,19,23].

In the present study, we investigated the effects of HDAC inhibitors on survival of dopaminergic neurons, since dopaminergic neurons are the most important target cells in PD. A single treatment with the HDAC inhibitor TSA resulted in a reduced survival and an increased apoptosis of cultured dopaminergic neuronal cells. TSA pre-treatment resulted in increased vulnerability of dopaminergic neurons to neurotoxic damage. The present study demonstrates for the first time that HDAC inhibitor reduced the survival and increased apoptosis in dopaminergic neuronal cell. Most importantly, HDAC inhibitor was found to exacerbate neurotoxic damage in dopaminergic neuronal cell. The dosages of HDAC inhibitor TSA used in the present studies are comparable to those in previous studies [5,23].

The activity of HDAC involves in determining the acetylation status of histone, thereby affecting the condensation status of the DNA, and hence gene transcription. HDAC catalyze the removal of acetyl groups from histone resulting in compaction of chromatin, thus silencing transcription probably by preventing the contact of transcription factors, regulatory complexes and RNA polymerases with the DNA. Inhibition of HDAC can relieve transcriptional repression [15]. Our results suggested that abnormal histone acetylation might play a role in PD pathogenesis.

The present results did not support the opinion that HDAC inhibition might be suitably neuroprotective PD therapy [4,5]. Actually, HDAC inhibitors have also been shown to induce apoptosis in Chen et al.'s report [5], despite of cell types. Other studies documented that VPA cause damage to astrocytes and decrease in the number of gliofilaments [25], even at very low doses [10]. The differing reported results might be due to the use of various cell types. Nevertheless, effects of HDAC inhibitors might be more complex than the neuroprotection in PD. Sechi et al. also suggested the importance of prudent use of VPA for patients with compromised astroglial functions as in AD [24].

Recent progress has suggested abnormal epigenetic modification such as HDAC dysregulation might play a role in neurodegenerative disease [1,14]. In previous studies, HDAC inhibitors mainly were tested as potential therapeutics for neurodegenerative disorders [1,14]. A role for HDAC in neurodegenerative disorders was first implied in Huntington’s disease (HD) [1]. In the mouse models of HD, treatment with HDAC inhibitors attenuate neuronal loss, increase motor function and extend survival. Although not as clearly defined as in HD, the neuropathology associated with Alzheimer’s disease (AD) might be due, at least in part, to HDAC dysregulation [1]. In other neurodegenerative disorders, roles of HDAC inhibitors have not been addressed clearly yet.
Fig. 3. TSA pre-treatment increased the neurotoxic effects of MPP⁺ and rotenone on dopaminergic neuronal cells. Cells were pre-treated with TSA, followed by treatment with MPP⁺ or rotenone. Cell viability assay was performed by trypan blue staining. These values represent mean ± SEM of triplicate experiments (n = 3). Columns, mean; bars, SEM. (A) TSA pre-treatment (10 nM, 12 h) increased the neurotoxic effects of MPP⁺ (200 μM, 12 h) on N27 cells. *P < 0.05, TSA vs. TSA/MPP; #P < 0.001, MPP vs. TSA/MPP. (B) TSA pre-treatment (10 nM, 12 h) increased neurotoxic effects of rotenone (0.5 μM, 12 h) on N27 cells. *P < 0.01, TSA vs. TSA/rotenone; #P < 0.05, rotenone vs. TSA/rotenone. (C) TSA pre-treatment (50 nM, 12 h) increased neurotoxic effects of MPP⁺ (400 μM, 24 h) on SH-SY5Y cells. *P < 0.01, TSA vs. TSA/MPP; #P < 0.01, MPP vs. TSA/MPP. (D) TSA pre-treatment (50 nM, 24 h) increased neurotoxic effects of rotenone treatment (1 μM, 12 h) on SH-SY5Y cells. *P < 0.001, TSA vs. TSA/rotenone; #P < 0.01, rotenone vs. TSA/rotenone. ctr: control; TSA: single TSA pre-treatment; rotenone: single rotenone treatment; TSA/MPP: TSA pre-treatment and then combined treatment with MPP⁺; TSA/rotenone: TSA pre-treatment and then combined treatment with rotenone.

By comparing previous findings with ours, it is suggested that effects of HDAC inhibitors might be multiple and complicated in nervous system rather than therapeutic. In some case, HDAC inhibitors could act as potential therapeutics for neurodegenerative disease. In other case, HDAC inhibitors might play a role in pathogenesis in neurodegenerative disease. This may depend on epigenetic status, cell type and tissue specificity. Therefore, usage of HDAC inhibitor in epigenetic therapy of human disease needs to be very prudent. Future studies to examine the efficacy of HDAC inhibitors in mouse models of PD and other neurodegenerative disease are needed.

Based on the present findings, HDAC inhibitors might promote dopaminergic neuronal cell death as a result of environmental neurotoxic factors. Therefore, more caution should be taken when using HDAC inhibitors as an epigenetic drug for the treatment of human diseases including a range of central nervous system disorders. Further understanding involved in the epigenetic process of PD is needed.

In summary, HDAC inhibitors might play a role in PD pathogenesis by contributing to dopaminergic neuron vulnerability to environmental neurotoxins. Moreover, more caution should be taken when using HDAC inhibitors as epigenetic drug in therapy for human diseases. Further studies are needed to address the mechanism of HDAC inhibitors acting in PD.

Acknowledgments

This work was supported by grants from the Chinese National Basic Research Program (2006CB500700) and the Beijing Natural Science Foundation (7082008).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2009.10.037.
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