Neuroprotective effects of saikosaponin-d on MPP⁺-induced cytotoxicity in SH-SY5Y cells via regulation of SIRT3

Jun Yang¹, Cheng-Yan Li¹, Gui-Bin Zhang²

¹Department of Neurology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, China; ²Department of Neurology, Xiangyang No. 1 People’s Hospital, Xiangyang Hospital Affiliated to Hubei University of Medicine, Xiangyang 441000, Hubei, China

Received September 17, 2015; Accepted March 2, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Saikosaponin-d (Ssd), one of the major components extracted from Bupleurum falcatum L exhibits a variety of pharmaceutical activities including anti-tumor, anti-inflammation and immunomodulatory properties. In the current study, we investigated the protective effects of Ssd on MPP⁺-induced cytotoxicity in SH-SY5Y cells along with the underlying mechanisms. Our study showed that Ssd exerted a concentration-dependent neuroprotective effect against MPP⁺-induced reduction of cell viability in the in vitro model of Parkinson’s disease. In addition, MPP⁺ exposure led to an increased oxidative stress, apoptotic cell deaths and caspase-3 activity, which was attenuated by incubation with Ssd. On the molecular level, we found that Ssd treatment significantly increased the mRNA and protein expression of SIRT3. Meanwhile, SIRT3 knockdown abolished the protective effects of Ssd on SH-SY5Y cells. Taken together, these results demonstrated that treatment of SH-SY5Y cells with Ssd could protect against MPP⁺-induced cytotoxicity via regulation of SIRT3 expression.

Keywords: Saikosaponin-d, Parkinson’s disease, oxidative stress, SIRT3

Introduction

Parkinson’s disease (PD) is one of the most common chronic neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the substantia nigra and subsequent depletion of dopamine in the striatum [1, 2]. Up to now, the etiology and pathogenesis of PD are not fully elucidated. Various surgical and pharmacological therapies have been used to treat patients with PD, but some of these have significant adverse effects [3]. In recent years, increasing studies have demonstrated that a part of active compounds extracted from herbal medicine have potential therapeutic effects on PD models both in vitro and in vivo [4-6].

Saikosaponin-d (Ssd) is one of the major triterpenoid saponins derived from Bupleurum falcatum L, which is a commonly prescribed agent against inflammatory and infectious diseases in China, Japan and other Asian countries [7]. Recent studies have shown that Ssd has anti-inflammatory, immune-modulatory, and antiviral activities, making it a potential chemotherapeutic drug for clinical application [8, 9]. However, the neuroprotective effects of Ssd and the underlying mechanism have rarely been investigated.

Currently, the neuronal toxins, such as 1-methyl-4-phenyl-pyridinium (MPP⁺), have been applied to investigate the PD [10]. MPP⁺, a metabolite of MPTP by monoamine oxidase-B, has been identified as a neurotoxin and widely used in both in vitro and in vivo PD models [11]. Using an in vitro PD model induced by MPP⁺, we evaluated the neuroprotective effects of Ssd as well as the molecular mechanism, aiming to identify a possible therapeutic application to PD.

Materials and methods

Cell culture and reagents

SH-SY5Y cells were grown in Dulbecco’s modified Eagle medium Ham’s F12 (Gibco, CA, USA) supplemented with 10% fetal bovine
Neuroprotective effects of saikosaponin-d

serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂/95% air incubator. Ssd was dissolved in culture medium at a concentration of 5 mM for a stock solution. For treatment of cells, Ssd was diluted in culture medium to the appropriate concentration. Ssd and MPP⁺ were purchased from Sigma (St. Louis, MO, USA).

Cell viability assay

The cell viability was measured by a quantitative colorimetric assay with MTT. To be brief, exponentially growing cells at a density of 5×10⁴ were seeded in a 96-well plate cells/well. After 24 h, SH-SY5Y cells were firstly subjected to a serial condition test with Ssd (15, 30, 45 μM) for 5 h and then with MPP⁺ for 24 h. The optical density was measured using a microplate reader at a wavelength of 450 nm. All readings were compared with the control, which represented 100% viability.

Apoptosis assay

Cellular apoptosis was monitored by the fluorescein isothiocyanate (FITC)/Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA) according to the manufacturer’s protocol. To be brief, SH-SY5Y cells at the density of 1×10⁵ cells/well were stained with FITC/Annexin V and propidium iodide (PI). Then, cell apoptosis was measured using a flow cytometry analyzer (BD Biosciences, San Jose, CA).

Caspase-3 activity assay

SH-SY5Y cells at the density of 1×10⁴ cells/well were treated with MPP⁺ and/or Ssd for 24 h, and caspase-3 activity was determined according to the instruction of caspase-3 colorimetric assay kit (St. Louis, MO, US), which is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. The proteolytic reaction was carried out in isolation buffer containing 50 μg of cytosolic protein and 50 μM specific caspase substrates. The reaction was measured by changes in absorbance at 405 nm using a 96-well plate reader.

Measurement of ROS production

Cells were incubated with 2,7-dichlorofluorescein diacetate (DCF-DA) (Sigma, St. Louis, MO, USA) for 30 min at 37°C in the dark. The plates were immediately washed with serum-free medium and covered with 200 μl of serum-free medium. After 24 h, cells were incubated with 20 μM DCF-DA for 1 h at 37°C in the dark. The optical density was measured using a microplate reader at a wavelength of 485 nm.

Figure 1. Effects of Ssd on MPP⁺-induced cytotoxicity in SH-SY5Y cells. A. SH-SY5Y cells were treated with different concentrations of Ssd (0, 15, 30, 45, 60 μM) for 24 h. Cell viability was determined with MTT assay. B. SH-SY5Y cells were exposed to a serial condition test with MPP⁺ (0, 0.1, 0.5, 1 and 1.5 mM) for 24 h. Cell viability was measured with MTT assay. C. SH-SY5Y cells were subjected to Ssd (0, 15, 30, 45 μM) combined with MPP⁺ (1 mM) for 24 h. Cell viability was determined with MTT assay. *P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control; #P<0.05 vs. MPP⁺.
Neuroprotective effects of saikosaponin-d

USA) for 1 h at 37°C in the dark, and then resuspended in PBS. Intracellular ROS production was assessed using a fluorescence microscope (OLYMPUS, Germany).

**Western blot**

Total cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on a nitrocellulose filter membrane. The membrane was incubated with primary antibodies against SIRT3 (1:1000 dilution) and GAPDH (1:5000 dilution). Horseradish peroxidase-conjugated secondary antibodies (1:10000) were applied on the membrane and the bound secondary antibody was detected with the enhanced chemiluminescence reagents (Pierce, Rockford, IL). All antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

**Statistical analysis**

Data are expressed as mean ± SD. The difference between groups was analyzed by ANOVA using the SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). A P value of < 0.05 was considered statistically significant.

**Results**

*Effects of Ssd on MPP⁺-induced cytotoxicity in SH-SY5Y cells*

To examine if Ssd affected the survival of SH-SY5Y cells, different concentrations of Ssd (0, 15, 30, 45, 60 μM) were added to the cells for 24 h. As shown in Figure 1A, low doses of Ssd (15–45 μM) had little effect on cell proliferation, whereas Ssd at high dose (60 μM) obviously inhibited the growth of SH-SY5Y cells. Consistent with previous studies, we found that treatment with MPP⁺ (0, 0.1, 0.5, 1 and 1.5 mM) caused a significant decrease in cell viability in a concentration-dependent manner (Figure 1B). Based on these data, we decided to use low doses of Ssd (15, 30, 45 μM) and MPP⁺ at 1 mM for the following experiments. To examine the protective effect of Ssd on MPP⁺-induced cell deaths, SH-SY5Y cells were exposed to different doses (15, 30, 45 μM) of Ssd 5 h prior to MPP⁺ (1 mM) treatment. The results of MTT assay showed that Ssd significantly increased the viability of the SH-SY5Y cells after MPP⁺ administration for 24 h (Figure 1C).

*Ssd inhibited MPP⁺-induced ROS accumulation in SH-SY5Y cells*

Oxidative stress is a well-established event in the pathology of many diseases, including PD. To examine whether the protective effect of Ssd against MPP⁺-induced cytotoxicity was attributed to its antioxidant activity, the intracellular ROS levels were measured in SH-SY5Y cells. As shown in Figure 2, we found that the MPP⁺ treatment significantly increased the ROS generation, whereas Ssd attenuated the intracellular ROS levels in a concentration-dependent manner. These data suggested that Ssd could reduce oxidative stress in SH-SY5Y cells.

*Ssd reduced MPP⁺-induced cell apoptosis in SH-SY5Y cells*

To examine the effects of Ssd on cell apoptotic deaths, SH-SY5Y cells were incubated with MPP⁺ alone or in combination with Ssd in different doses. Flow cytometry analysis showed that Ssd treatment significantly inhibited MPP⁺-induced cell apoptosis in a dose-dependent fashion (Figure 3A). Moreover, the activity of caspase-3 was examined in order to evaluate the apoptosis process following Ssd treatment. Following 24 h treatment of SH-SY5Y cells with MPP⁺, the caspase-3 activity was dramatically increased nearly three folds. However, Ssd administration attenuated the MPP⁺-induced caspase-3 activation in a dose-dependent manner (Figure 3B). These results indicated...
that Ssd could alleviate cell apoptosis induced by MPP⁺ in SH-SY5Y cells.

**Up-regulation of SIRT3 by Ssd administration in SH-SY5Y cells**

We further investigated the molecular mechanism underlying the protective effects of Ssd on SH-SY5Y cells. Since SIRT3 plays important roles in cell survival and apoptosis, we hypothesized that SIRT3 may be involved in the neuroprotective effects of Ssd in SH-SY5Y cells. Data from qRT-PCR indicated that SH-SY5Y cells exposed to MPP⁺ alone significantly decreased SIRT3 mRNA level. However, a dramatic increase of SIRT3 mRNA expression was detected in Ssd-treated SH-SY5Y cells (**Figure 4A**). Similarly, the protein level of SIRT3 was reduced after MPP⁺ treatment, but was enhanced in Ssd-treated cells (**Figure 4B**). These data suggested that Ssd promoted the expression of SIRT3 in SH-SY5Y cells.
Neuroprotective effects of saikosaponin-d

Ssd protected SH-SY5Y cells against MPP+-induced cytotoxicity via SIRT3

In order to determine whether SIRT3 is involved in the protective effects of Ssd, we down regulated SIRT3 with a specific small interfering RNA (siRNA) in SH-SY5Y cells. The expression of SIRT3 was obviously reduced both at mRNA and protein levels (Figure 5A and 5B). Consequently, our data showed that Ssd failed to promote SH-SY5Y cells viability after treatment with MPP+ (Figure 5C). In addition, SIRT3 knockdown in SH-SY5Y cells exhibited no significant differences in ROS production (Figure 5D) and cell apoptosis (Figure 5E) with or without Ssd treatment. These results demonstrated that SIRT3 knockdown reversed the protective effects of Ssd on SH-SY5Y cells.

Discussion

Parkinson’s disease is a movement impediment characterized by dopaminergic degeneration and dysfunction. Up to now, the pathogenesis of PD has been not fully elucidated and increasing interest has been devoted to the treatment or prevention of PD by herbal medicines [12]. Our study showed that Ssd exerted a concentration-dependent neuroprotective effect against the MPP+ induced cell viability reduction and apoptotic deaths in the in vitro model of PD. At the molecular level, we found that the protective effects of Ssd were, at least in part, mediated by regulation of SIRT3.

Growing evidences indicate that oxidative stress is involved in the pathogenesis of neurodegenerative diseases such as PD [13-15]. A number of ROS-generating pathways including glycolysis, NAD(P)H oxidase, xanthine oxidase, and uncoupling of nitric oxide synthase have been identified as potential contributors to the development and progression of PD [16]. Furthermore, it is suggested that ROS accumulation participated in the neurotoxicity induced...
by MPP+ and might contribute to the apoptotic death in PD [17, 18]. A recent study has reported that total saikosaponins of Bupleurum yin-chowense exhibits a neuroprotective effects on corticosterone-induced apoptosis using an in vitro model of depression [19]. In the current study, we investigated the neuroprotective effect of Ssd in SH-SY5Y cells treated with MPP+. Our results suggested that Ssd treatment significantly reduced the MPP+-induced neurotoxicity in SH-SY5Y cells. In addition, MPP+ exposure dramatically elevated the ROS accumulation, cell apoptosis and caspase-3 activity, which were efficiently attenuated by Ssd treatment. These results demonstrated that Ssd exhibited strong neuroprotective effects against MPP+-induced cytotoxicity.

The sirtuins are a conserved family of class III histone deacetylases, which are involved in transcriptional regulation, genetic control of aging, and longevity of organisms ranging from yeasts to humans [20-22]. Among the known sirtuin members, SIRT3 is emerging as a pivotal regulator of cell proliferation and oxidative stress [23]. A recent study has shown that SIRT3 contributes to the repair of mitochondrial DNA and protects from apoptotic cell death under oxidative stress [24]. Another study demonstrated that SIRT3 protects cortical neurons against oxidative stress via regulating mitochondrial Ca2+ and mitochondrial biogenesis [25]. In the present study, we determined to explore whether SIRT3 plays a critical role in MPP+-induced cytotoxicity in SH-SY5Y cells. Interestingly, MPP+ incubation led to a significant reduction in SIRT3 expression both at the mRNA and protein levels. However, such changes were reversed by Ssd pretreatment, suggesting the regulatory role of Ssd in SIRT3 levels. Furthermore, we failed to observe obvious changes in cell viability, apoptosis and ROS production after downregulation of SIRT3 with specific SIRT3 siRNA in SH-SY5Y cells. These results suggested that Ssd protected SH-SY5Y cells against MPP+-induced cytotoxicity via regulation of SIRT3 expression.

In summary, our current study for the first time demonstrates that Ssd has neuroprotective effects against MPP+-induced cytotoxicity possibly through regulation of SIRT3 expression. Our data suggest that Ssd may be a potential therapeutic agent for neurodegenerative diseases such as PD.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cheng-Yan Li, Department of Neurology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan 430060, Hubei, China. Tel: 86-27-88041911; Fax: 86-27-88041911; E-mail: lcy@medmail.com.cn

References

Neuroprotective effects of saikosaponin-d


