ARTICLE
Journal of Cellular Biochemistry 111:1537–1545 (2010)

Journal of Cellular Biochemistry

Rg1 Protects Iron-Induced Neurotoxicity Through Antioxidant and Iron Regulatory Proteins in 6-OHDA-Treated MES23.5 Cells

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ABSTRACT

Ginsenoside-Rg1 is one of the pharmacologically active components isolated from ginseng. It was reported that Rg1 protected dopamine (DA) neurons in 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease (PD) models in vivo and in vitro. Our previous study also demonstrated that iron accumulation was involved in the toxicity of 6-OHDA. However, whether Rg1 could protect DA neurons against 6-OHDA toxicity by modulating iron accumulation and iron-induced oxidative stress is not clear. Therefore, the present study was carried out to elucidate this effect in 6-OHDA-treated MES23.5 cells and the possible mechanisms were also conducted. Findings showed Rg1 restored iron-induced decrease in mitochondrial transmembrane potential in MES23.5 cells, and increased ferrous iron influx was found in 6-OHDA-treated cells. Rg1 pretreatment could decrease this iron influx by inhibiting 6-OHDA-induced up-regulation of an iron importer protein divalent metal transporter 1 with iron responsive element (DMT1 + IRE). Furthermore, findings also showed that the effect of Rg1 on DMT1 + IRE expression was due to its inhibition of iron regulatory proteins (IRPs) by its antioxidant effect. These results suggested that the neuroprotective effect of Rg1 against iron toxicity in 6-OHDA-treated cells was to decrease the cellular iron accumulation and attenuate the improper up-regulation of DMT1 + IRE via IRE/IRP system. This provides new insight to understand the pharmacological effects of Rg1 on iron-induced degeneration of DA neurons. J. Cell. Biochem. 111: 1537–1545, 2010.

KEY WORDS: ANTIOXIDANT; RG1; IRON REGULATORY PROTEIN (IRP); FERROUS IRON; 6-HYDROXYDOPAMINE (6-OHDA)

Parkinsons's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra (SN), giving rise to dopamine (DA) depletion in the striatum. Although the exact causes of PD are not known, genetic mutations, oxidative stress, mitochondrial, ubiquitin–proteasome system dysfunction, and environmental factors are considered to be involved. Recently, there is increasing evidence that the onset of PD is associated with iron accumulation in substantia nigra pars compacta (SNpc) in parkinsonian brain [Youdim et al., 2004; Berg et al., 2006; Jiang et al., 2006, 2007; Wang et al., 2007]. Because DA neurons of SN show an exceptionally high degree of vulnerability to reactive oxygen species (ROS), excessive nigral iron-induced oxidative stress by Fenton reaction in these DA neurons could lead neuronal death and was thought to be factors in the pathogenesis of PD [Qian et al., 1997; Bostanci and Bagirici, 2008]. This has led to the current notion that drugs against iron accumulation might be effective.

Ginsenosides or ginseng saponins, the main active components of ginseng (the root of Panax ginseng C.A. Meyer, Araliaceae) have been accumulatively reported to exert neuroprotective effects in the central nervous system [Lee et al., 2002; Gao et al., 2009; Wang et al., 2009; Xu et al., 2009]. There are two major classes of ginsenosides, namely protopanaxatriol (Rg1, Rg2, Re, Rf, and Rh1) and protopanaxadiol (Rb1, Rb2, Rc, Rd, Rg3, Rh2, and Rh3). Among

Abbreviations used: SN, substantia nigra; SNpc, substantia nigra pars compacta; PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; DMT1, divalent metal transporter 1; Nramp2, natural resistance-associated macrophage protein 2; IRE, iron responsive element; IRP, iron regulatory protein.

Grant sponsor: National Natural Science Foundation of China; Grant number: (30870858, 30930036); Grant sponsor: Natural Science and Fund of Shandong Province for Distinguished Young Scholars; Grant number: JQ200807.

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Received 11 July 2010; Accepted 7 September 2010 • DOI 10.1002/jcb.22885 • © 2010 Wiley-Liss, Inc.
Published online 24 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

them, ginsenoside Rg1 is one of the most active and abundant compounds found in ginseng [Liu and Xiao, 1992]. It was reported that Rg1 protected DA neurons against glutamate [Radad et al., 2004b], rotenone [Leung et al., 2007], 1-methyl-4-phenylpyridinium (MPP^+) [Radad et al., 2004a] and 6-hydroxydopamine (6-OHDA) toxicities [Xu et al., 2008, 2009c; Gao et al., 2009; Ge et al., 2010], indicating the protective effect of Rg1 on DA neurons in PD. However, whether Rg1 could protect DA neurons by modulating iron accumulation, which plays a key role in PD, and the underlying mechanisms are not clear.

6-OHDA is a widely used neurotoxic agent that can selectively damage dopaminergic neurons in vivo and in vitro. The toxicity of 6-OHDA is mediated through generation of ROS, oxidative damage of mitochondria. Our previous study demonstrated that iron accumulation was involved in the toxicity of 6-OHDA [Song et al., 2007]. And we also observed a ferrous iron importer divalent metal transporter 1 (DMT1) might account for nigral iron accumulation in PD. DMT1 is a ferrous iron importer [Gunshin et al., 1997]. Its mRNA encodes four proteins which differs in both the C-terminus [with iron responsive element (IRE) and without IRE] and the N-terminus (two different promoters) [Lee et al., 1998; Hubert and Hentze, 2002; Mackenzie et al., 2007]. In our previous study, we demonstrated that in the SN of 6-OHDA-induced PD rats and MES23.5 cells, DMT1 + IRE was up-regulated. This has also been confirmed by [Salazar et al., 2008] who demonstrated that DMT1 + IRE expression and iron levels were found to be increased in the SNpc of PD patients when compared with that of age-matched controls, suggesting that increased DMT1 + IRE expression might account for nigral iron accumulation in 6-OHDA-induced PD models. It was acceptable that the regulation of iron-related proteins occurs at multiple levels, post-transcriptional control mediated by the IRE/iron regulatory protein (IRP) system has emerged as a central regulatory mechanism. The regulating of iron importer DMT1 + IRE has been proved to be dependent on IRE/IRP system [Lee et al., 1998]. In our previous experiment, we also showed that DMT1 + IRE up-regulation in 6-OHDA-induced PD models occured in an IRE/IRP-dependent manner and that it was initiated by 6-OHDA-induced intracellular oxidative stress [Jiang et al., 2010]. However, whether Rg1 could regulate the expression of iron transporter DMT1 + IRE and IRE/IRP system to exert its protective effect on iron accumulation is not elucidated. Therefore, in the present study 6-OHDA was used to investigate the protective effect of Rg1 on iron accumulation in MES23.5 dopaminergic neurons and the possible mechanisms.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis. MO). The primary DMT1 + IRE and IRPs antibodies were purchased from the ADI (ADI, San Antonio, TX). Calcein-AM was from Molecular Probes Inc (Carlsbad, CA). Dulbecco's modified Eagle's medium Nutrient Mixture-F12 (DMEM/F12) were from Gibco (Grand Island, NY). Rg1 was purchased from Baiqiuen Medical University. Other chemicals and regents available were from local commercial sources.

CELL CULTURE

MES23.5 cell line offered by Dr. Wei-Dong Le (Baylor College of Medicine, TX) is a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons, which exhibits several properties similar to the primary neurons originated in the SN [Crawford et al., 1992]. They were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% FBS, 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37°C, in a humid 5% CO_2, 95% air environment. Ginsenoside-Rg1 was dissolved in absolute alcohol as a concentrated stock and further diluted to their final concentration in external recording solution. For experiments, cells were seeded at a density of $1 \times 10^5/cm^2$ in the plastic flasks or on glass cover slips. MES23.5 cells were treated with reagents as follows: MES23.5 cells were incubated with 100 μmol/L ferrous iron for 3 h for the iron incubation experiments. For 6-OHDA treatment, MES23.5 cells were treated with 6-OHDA (10 μmol/L) for 24 h. For pretreatment, MES23.5 cells were pretreated with 10^{-6} mol/L or 10^{-7} mol/L Rg1 for 24 h before 6-OHDA (10 μmol/L) treatment.

TOTAL RNA EXTRACTION AND QUANTITATIVE AND SEMIQUANTITATIVE PCR

Total RNA was isolated by using Trizol Reagent from MES23.5 cells according to the manufacturer's instructions. Then 2 μg of total RNA was reverse-transcribed in a 20 μl reaction using reverse-transcription system. Primers were designed using computer software (Primer Premier 5.0). Quantitative PCR was employed to detect the changes of DMT1 + IRE. TaqMan probe and the primers were designed to sequences using the default settings of Primer Express 2.0 (PE Applied Biosystems). Each set of primers was used with a TaqMan probe labeled at the 5-end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3-end with the 6-carboxy-tetramethylrhodamine (TAMRA) quencher dye. The following primers and probes were employed for DMT1 + IRE: forward 5′-TGGCTGTCACGAGTGCTTACA-3′, reverse 5′-CCATGGCCTTGGACAGCTATT-3′, probe 5′-TTACCCTGTAGCATTAGGCAGCACC-3′; Rat GAPDH gene was used as the reference: forward 5′-CCCCCAATGTATCCGTTGTG-3′, reverse 5′-GTAGCCCAGGATGCCCTTTAGT-3′, probe 5′-TCTGACATGCCGCCTGGAGAAACC-3′. Reactions were carried out on an ABI PRISM® 7500 Sequence Detection System using the relative quantification option of the SDS1.2.1 software (Applied Biosystems). Each reaction was run in triplicate with 2 μl sample in a total volume of 20 μl with primers and probes to a final concentration of 0.25 μmol/L. A passive reference dye ROXII, was used to normalize the reporter signal. Amplification and detection were performed with the following conditions: an initial hold at 95°C for 10 s followed by 40 cycles at 95°C for 5 s and 60°C for 45 s.

As for IRPs, semiquantitative PCR was used. Rat IRP1: forward primer, 5′-TTACCAAGCACCTCCGACAA-3′, backward primer, 5′-AATCCTGCGCCTAACATCA-3′. The amplified IRP1 fragment was 562 bp. We amplified 622 bp with the primer of Rat IRP2 (forward primer, 5′-AGCCGAAACTCAGGAACA-3′, backward primer, 5′-TCACATTGTCAACAGGGAA-3′). Mouse GAPDH was also used: forward primer, 5′-TTCACCACCATGGAGAAGGC-3′; backward primer, 5′-GGCATGGACTGTG GTCATGA-3′. The amplified GAPDH fragment was 236 bp. The samples were heated at 95°C for 5 min and

the subsequent cycles were performed at three temperature steps as followed: IRP1; 94°C for 30 s, 62°C for 30 s, 72°C for 45 s for 30 cycles; IRP2: 94°C for 30 s, 50°C for 30 s, 72°C for 45 s for 35 cycles, then an additional 10 min for extension was carried out; Ethidium bromide stained gels were scanned and qualified using Tanon Image Software.

WESTERN BLOTS

Cells were lysed directly on the culture dishes using lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA) plus 1 mM PMSF and protease inhibitors (pepstatin 1 μg/ml, aprotinin 1 μg/ml, leupeptin 1 μg/ml). The protein concentration was determined by the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA). Sixty micrograms total proteins were separated using 10% SDS–PAGE and then transferred to PVDF membranes. After overnight blocking with 5% non-fat milk at 4°C, the membranes were incubated with rabbit anti-rat DMT1 + IRE antibody (1:2,000, ADI), IRP1 or IRP2 polyclonal antibody (ADI, 1:2000) for 1 h at room temperature. Anti-rabbit secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1:500. Cross-reactivity was visualized using ECL Western blotting detection reagents and then was analyzed through scanning densitometry by Tanon Image System. β-actin was detected by rabbit anti-β-actin monoclonal antibody (1:10,000, Sigma) according to similar procedures to ensure equal sample protein loading.

CALCEIN LOADING OF CELLS AND FERROUS IRON INFLUX ASSAY

Calcein-AM is a membrane-permeative, non-fluorescent molecule that becomes fluorescent upon intracellular cleavage by cytoplasmic esterases to calcein (which is membrane impermeative). It is pH-independent and stable and can be quenched rapidly by divalent metals and reversed easily by the chelators [Tenopoulou et al., 2005; Song et al., 2010]. Ferrous iron influx into MES23.5 cells was determined by the quenching of calcein fluorescence [Wetli et al., 2006; Song et al., 2007; Zhang et al., 2009]. Cells were incubated with calcein-AM (0.5 μmol/L final concentration) in HEPES-buffered saline (HBS, 10 mmol/L HEPES, 150 mmol/L NaCl, pH = 7.4) for 30 min at 37°C. The excess calcein on cell surface was washed out three times with HBS. The coverslips were mounted in a perfused heated chamber. Calcein fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths and fluorescence intensity was measured every 3 min for 30 min while perfusing with 1 mM ferrous iron (ferrous sulfate in ascorbic acid solution, 1:44 molar ratio) [Picard et al., 2000], prepared immediately prior to the experiments. Ascorbic acid maintained the reduced status of ferrous iron, in addition, ascorbate acted as a chelator to maintain the iron in solution. The mean fluorescence signal of 25–30 single cells in four separate fields was monitored at 200× magnification and processed with Fluoview 5.0 Software.

REACTIVE OXYGEN SPECIES (ROS) ASSAY

The production of superoxide and hydrogen peroxide (H_2O_2) was assessed by the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma) as described before [Chwa et al., 2006; Alvira et al., 2007; Xu et al., 2010]. Cells were washed three times with PBS, and then incubated in DMEM/F12 containing H_2DCF-DA (10 μmol/L) for 30 min. The fluorescence signals were measured with excitation filter of 488 and 525 nm emission wavelengths (Fluorescence 1, FL1).

FLOW CYTOMETRIC MEASUREMENT OF MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ($\Delta\Psi$M)

ΔΨm in MES23.5 cells were measured by flow cytometry (Becton Dickinson) with rhodamine123 dye as described before [Zhu and Liu, 2004; Sanelli et al., 2007]. Cells were treated with 100 μmol/L ferrous iron (pH = 6.0) for 4 h, and then were incubated with rhodamine123 dye in a final concentration of 5 μmol/L for 30 min at 37°C. After washing twice with PBS, fluorescence was recorded at 488 nm excitation and 525 nm emission wavelengths (FL1).

STATISTICAL ANALYSIS

Each experiment was performed at least three times, and the results are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare the differences between means. Influx studies were carried out by the two ways ANOVA and data were presented as mean ± SEM. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

RG1 RESTORED IRON-INDUCED DECREASE IN $\Delta\Psi$M

Iron-induced oxidative stress plays a critical role in pathology of PD. Excess intracellular iron could induce cell damage including mitochondrial dysfunction by Fenton reaction. Changes in the ΔΨm are makers of the mitochondrial function. To clarify the protective effect of Rg1 on iron-induced cell damage, changes of ΔΨm were detected in this study. Results showed that iron treatment decreased ΔΨm. Rg1 pretreatment could restore ΔΨm to the control (Fig. 1). This indicated Rg1 could protect iron-induced damage of DA neurons.

RG1 TREATMENT PREVENTED FERROUS IRON INFLUX IN 6-OHDA-TREATED MES23.5 CELLS

Increased iron influx was found to be involved in the neurotoxicity of 6-OHDA, indicating iron-induced cell damage contributed to the DA degeneration in PD cell models (6-OHDA). To make sure the effect of Rg1 on 6-OHDA-induced increase in iron influx, we detected the iron influx function. To avoid influence of cell loss, incubation with 10 μmol/L 6-OHDA for 24 h was employed. Results showed there was a significant decrease in the fluorescence intensity in 6-OHDA-treated cells compared to the control when perfused with ferrous iron. This indicated more ferrous iron entered into 6-OHDA-treated cells compared to the control. Pretreated with 10^{-6} mol/L or 10^{-7} mol/L Rg1 restored the fluorescence intensity to the control levels (Fig. 2). This suggested that 6-OHDA could increase ferrous iron uptake in MES23.5 cells and this could be prevented by pretreatment with Rg1.

THE EFFECT OF RG1 ON IRON INFLUX WAS DUE TO THE MODULATION OF DMT1 + IRE EXPRESSION IN 6-OHDA-TREATED MES23.5 CELLS

DMT1 is a ferrous iron importer protein. Our previous study demonstrated that the up-regulation of DMT1 + IRE contributed to iron accumulation in 6-OHDA-treated MES23.5 cells. This provides the hypothesis that the changes in iron influx function induced by

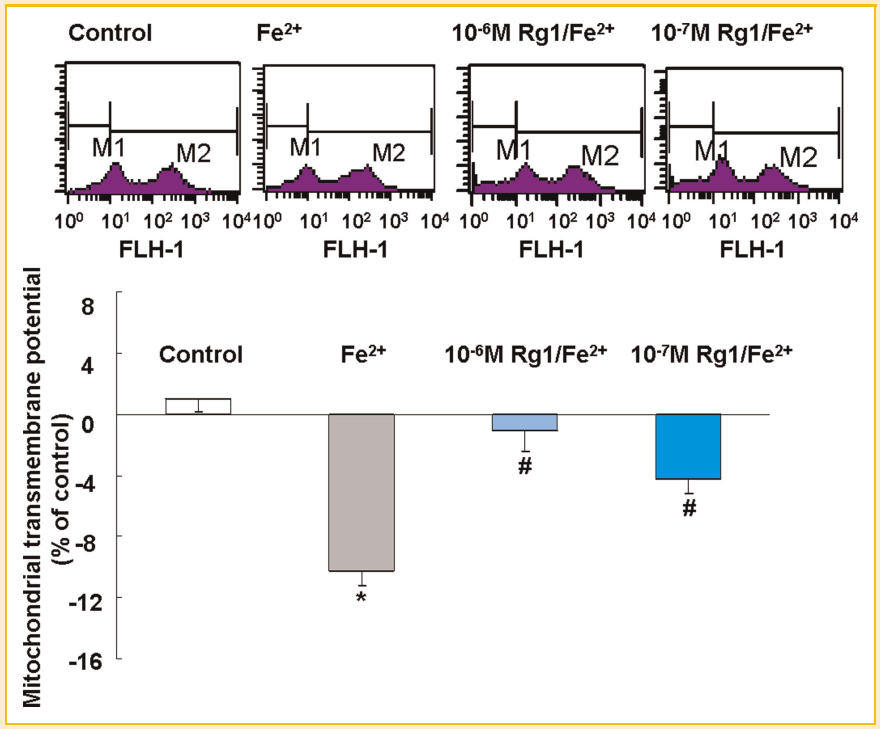

Fig. 1. Rg1 pretreatment restored iron-induced decrease in $\Delta\Psi m$. We detected the $\Delta\Psi m$ after iron treatment in MES23.5 cells. Results demonstrated that after iron incubation, $\Delta\Psi m$ decreased compared with control, and Rg1 could restore the $\Delta\Psi m$ to the control. Results were demonstrated as FL1-H (Fluorescence 1-histogram); setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellguest Software. %Gated for M2 was used to analysis the changes of the fluorescence. The control was set to 100% ($^{*}P < 0.01$, compared to control; $^{\#}P < 0.01$, compared to Fe^{2+} group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

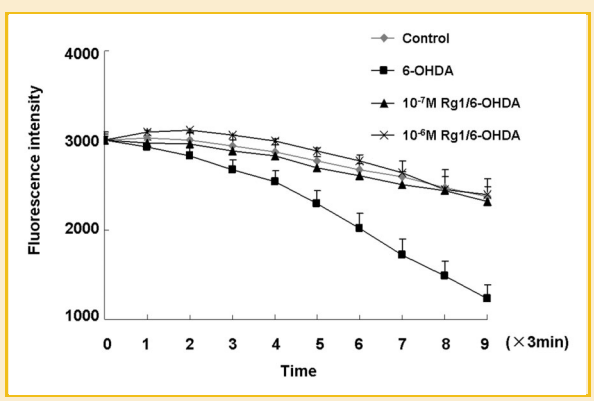

Fig. 2. Rg1 pretreatment suppressed calcein-indicated ferrous iron influx in 6-OHDA-treated MES23.5 cells. Ferrous iron influx function was detected by confocal using calcein fluorescence, which is an indicator of intracellular iron level. When perfusing with ferrous iron, fluorescence intensity in 6-OHDA-treated cells decreased more rapidly compared with the control. This indicated more ferrous iron entered into 6-OHDA-treated cells compared with the control, and there was a significant increase of fluorescence intensity in Rg1 pretreated cells compared with solely 6-OHDA-treated cells. This suggested that 6-OHDA could increase ferrous iron uptake in MES23.5 cells and this could be prevented by pretreatment with Rg1 pretreatment (two-way ANOVA, $P < 0.05$, cells treated with 6-OHDA vs. control; $P < 0.05$, cells pretreated with Rg1 vs. cells treated with 6-OHDA). Data were presented as mean ± SEM of six independent experiments.

Rg1 might due to its regulation on DMT1 + IRE expression. To investigate this possible effect, we measured DMT1 + IRE mRNA levels by real-time fluorescence quantitative PCR. Results showed there was a 2.41-fold increase of DMT1 + IRE mRNA in 6-OHDA-treated cells. Pretreatment with 10^{-6} mol/L or 10^{-7} mol/L Rg1 reversed the increase of DMT1 + IRE mRNA (Table I), and DMT1 + IRE protein levels were also detected in this study by Western blots. As showed in Figure 3, DMT1 + IRE protein in 6-OHDA-treated MES23.5 cells was up-regulated compared to the control, while pretreated with 10^{-6} mol/L or 10^{-7} mol/L Rg1 could inhibit this improper up-regulation of DMT1 + IRE protein (Fig. 3). This indicates Rg1 could attenuate the increased iron influx induced by 6-OHDA by modulating the expression of DMT1 + IRE.

RG1 REGULATED DMT1 + IRE EXPRESSION BY IRE/IRP SYSTEM IN 6-OHDA-TREATED MES23.5 CELLS

The presence of the IRE in the 3′-untranslated regions of DMT1 + IRE provides a site for binding of IRPs. IRPs are RNA-binding proteins that affect the translation and stabilization of specific mRNAs by binding to IREs in iron-related proteins and maintain the iron levels. This provided a hypothesis that Rg1 might suppress the expression of DMT1 + IRE by inhibiting the expression of IRPs. To further verify this hypothesis, the mRNA and protein levels of IRPs (IRP1 and IRP2) were measured by RT-PCR and Western blots. As expected, both IRP1 and IRP2 were up-regulated in mRNA (Fig. 4A,B) and protein levels (Fig. 5A,B) after 6-OHDA

TABLE I. Real-Time PCR Analysis for DMT1 + IRE mRNA Expression in MES23.5 Cells

Groups	DMT1 + IRE
Control	1 ± 0.23
6-OHDA	$2.41 \pm 0.36^{*}$
6-OHDA + Rg1 (10^{-6} M)	$1.07 \pm 0.10^{\#}$
6-OHDA + Rg1 (10^{-7} M)	$1.01 \pm 0.09^{\#}$

Data were presented as mean ± SEM. $F = 9.235$ ($^{*}P < 0.05$, compared with the control; $^{\#}P < 0.05$, compared with 6-OHDA-treated cells).

treatment compared to the control. When pretreated with 10^{-6} mol/L or 10^{-7} mol/L Rg1, the mRNA and protein levels of IRPs restored to the levels of control. This suggested that Rg1 could regulate DMT1 + IRE expression by modulating the expression of IRP1 and IRP2 on mRNA and protein levels in MES23.5 cells.

THE EFFECT OF RG1 ON 6-OHDA-INDUCED ROS GENERATION

6-OHDA is a neurotoxin to produce PD models, which could create oxidative stress through the production of H_2O_2 and other ROS. Accumulating evidence demonstrates that the generation of ROS might be involved in the regulation of the expression of IRPs and the subsequently regulate the iron-related proteins [Pantopoulos et al., 1996; Hanson and Leibold, 1999; Mladenka et al., 2006]. This provided a hypothesis that Rg1 might suppress the expression of IRPs by inhibiting ROS generation. We then measured the ROS production in MES23.5 cells by a cell-permeable ROS-sensitive fluorescent dye H_2DCFDA. As showed in Figure 6, the levels of ROS in 6-OHDA-treated MES23.5 cells was significantly increased compared with control, pretreatment with Rg1 (10^{-6} mol/L or 10^{-7} mol/L) decreased 6-OHDA-induced ROS production (Fig. 6). This indicated that Rg1 could inhibit the production of ROS by its antioxidant and this might contribute to the effect of Rg1 on the expression of IRPs.

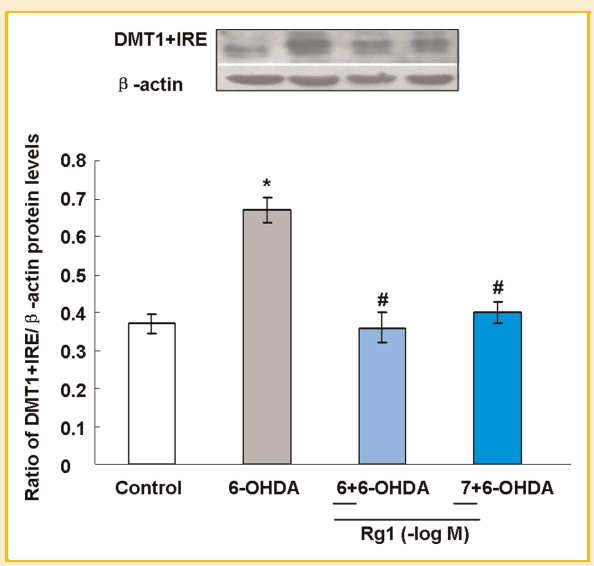

Fig. 3. Rg1 pretreatment attenuated up-regulation of DMT1 + IRE in 6-OHDA-treated MES23.5 cells. The protein levels of DMT1 + IRE was detected by Western blots. The protein levels of DMT1 + IRE in 6-OHDA-treated cells increased significantly compared with control ($^{*}P < 0.01$, compared with control). When pretreated with Rg1, the protein levels of DMT1 + IRE decreased compared with 6-OHDA-treated cells ($^{\#}P < 0.01$, compared with 6-OHDA-treated cells). Data were presented as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

Iron is the most abundant transitional metal in the brain and plays a critical role in maintaining normal brain function. However, increased levels of iron, especially Fe^{2+}, enhance the conversion of H_2O_2 by the Fenton reaction and favor a greater turnover in the Haber–Weiss cycle, resulting in an amplification of oxidative stress. Increased nigra iron levels have been found in PD patients and PD model in many studies [Youdim et al., 2004; Jiang et al., 2007; Salazar et al., 2008]. For PD patients this increase is mainly due to a rise in insoluble ferric iron (Fe^{3+}), rather than soluble ferrous iron (Fe^{2+}) [Sofic et al., 1988]. Fe^{3+} is the prevalent form of iron in the biological fluids. However, non-haem iron (Fe^{3+}) could be reduced by a ferric reductase (duodenal cytochrome b, Dcytb) to Fe^{2+} in the duodenum and be transported into cells by DMT1 [McKie et al., 2000]. It was also reported that the presence of stromal cell-derived receptor (SDR2), a homolog of Dcytb, which is predicted to act as a ferric reductase in neurons of the SN indicating SDR2 might serves as a ferric reductase to facilite Fe^{2+} transport in neurons [Ponting, 2001]. Fe^{2+} then could react with H_2O_2 to generate hydroxyl radical (OH·) and Fe^{3+} by Fenton reaction. This reaction may account both for the increase in ferric iron and also for the increased production of ROS in the SN in PD. Therefore, ferrous iron, which is responsible for the generation of ROS, was used in this study. Rg1 has been reported to protect DA neurons by reducing oxidative stress [Chen et al., 2003]. This indicated the possible protective effect of Rg1 on iron-induced oxidative stress. As iron could induce cell damage including mitochondrial dysfunction by oxidative stress and changes in the $\Delta\Psi$m are makers of the mitochondrial function. In this study, we first investigated the iron-induced changes in the $\Delta\Psi$m. Results showed Rg1 pretreatment could restore iron-induced decrease in $\Delta\Psi$m to the control. This suggested that Rg1 has the protective effect on iron-induced cell damage and might be a protective factor in the treatment of iron-induced degeneration of DA neuron in PD.

6-OHDA is a neurotoxin commonly used for modeling PD. 6-OHDA-treated animals exhibit the major hallmarks of PD, including loss of dopaminergic neurons in the SN, and iron levels are increased in SNpc and striatum after 6-OHDA injection [Hall et al., 1992]. It has been reported that 6-OHDA-induced neuron degeneration involves the processing of hydrogen peroxidase and OH· in the presence of ferrous iron by Fenton reaction [Sachs and Jonsson, 1975]. Our previous study have found increased intracellular iron levels were involved in the neurotoxicity of 6-OHDA. In this study, we demonstrated Rg1 pretreatment could prevent 6-OHDA-induced increase in iron levels in MES23.5 cells. Then what are the mechanisms underlying this decrease in iron levels caused by Rg1? It was reported that iron chelators could decrease intracellular iron levels by chelating iron. However, we have reported before that Rg1 had no iron chelating activity, indicating that Rg1 could not chelate

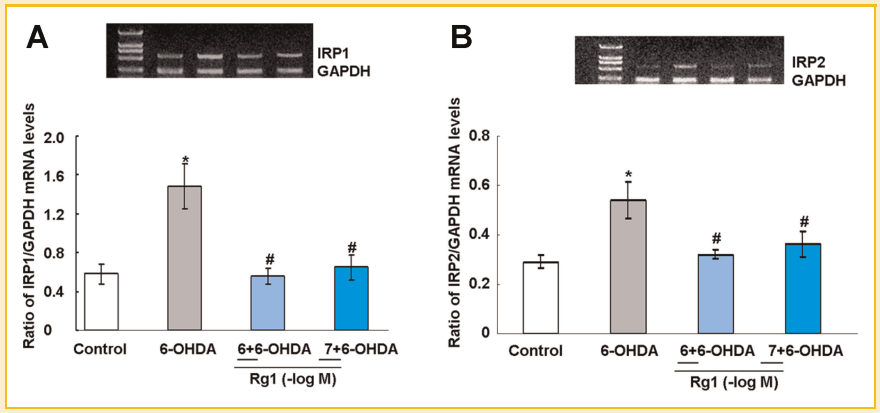

Fig. 4. Rg1 prevented the expression of IRP1 and IRP2 in mRNA levels in MES23.5 cells. The mRNA levels of IRP1 (A) and IRP2 (B) were detected by RT-PCR. Results showed both IRP1 and IRP2 were up-regulated in mRNA levels after 6-OHDA treatment compared with the control ($^{*}P < 0.05$, compared with the control). When pretreated with Rg1, the mRNA levels decreased significantly compared with 6-OHDA-treated cells and restored to the levels of the control ($^{\#}P < 0.05$, compared with 6-OHDA-treated cells). Data were presented as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

iron directly to decrease the iron levels [Wang et al., 2009]. This provides evidence that Rg1 might decrease intracellular iron levels by other mechanisms.

The intracellular iron increase might due to the increase iron uptake by iron transporters. Until now, DMT1 is thought to be one of endogenous transporters of ferrous iron. Our previous study also found iron importer DMT1 + IRE, but not DMT1 − IRE, was up-regulated, accompanied by increased iron staining in this region in vivo in the SN of 6-OHDA-induced PD rats and in vitro [Jiang et al., 2010]. This is consistent with the results reported recently that DMT1 + IRE increased in PD patients [Salazar et al., 2008]. This suggests that the up-regulation of DMT1 + IRE plays a critical role in iron accumulation in PD by increasing cellular iron uptake and Rg1 might decrease intracellular iron levels caused by 6-OHDA though modulating the expression of DMT1 + IRE. In the present study, our data showed that there was a significant increase of DMT1 + IRE mRNA and protein levels in 6-OHDA-treated MES23.5 dopaminergic cells and a decrease in Rg1 pretreated cells. This also explained why iron levels increased in 6-OHDA-treated cells and decreased in Rg1 pretreatment.

Another question is what is the mechanism underlying the inhibition of Rg1 on DMT1 + IRE up-regulation? IRPs are critical components of a sensory and regulatory system that are required to maintain iron homeostasis. There are two distinct forms of IRPs, IRP1 and IRP2. They are RNA-binding proteins, which could affect the translation and stabilization of specific mRNAs via binding to stem-loop structures known as IREs in iron-related proteins [Hentze and Kuhn, 1996; Eisenstein, 2000], indicating the critical role of IRPs in iron balance in the brain. In our previous experiment, we showed that DMT1 + IRE up-regulation in 6-OHDA-induced PD models occured in an IRE/IRP-dependent manner. This makes it possible that Rg1 could inhibit the up-regulation of DMT1 + IRE by regulating the expression of IRPs. As expected, we found both IRP1 and IRP2 were up-regulated in mRNA and protein levels after 6-OHDA treatment compared to the control. Rg1 pretreatments restored the mRNA and protein levels of IRPs to the levels of control.

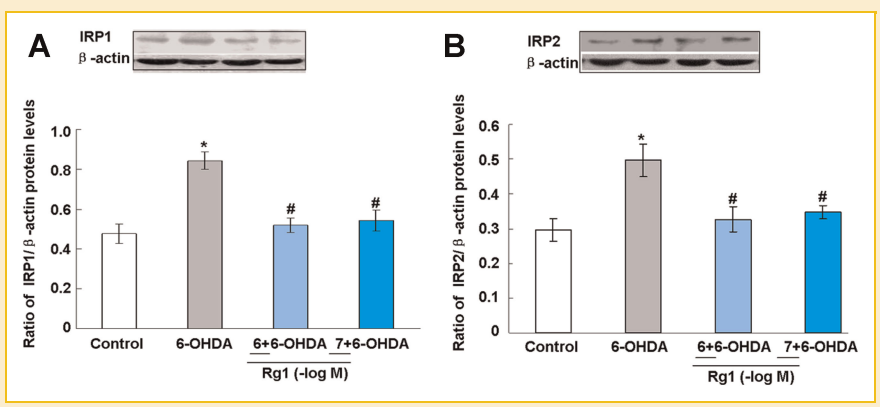

Fig. 5. Rg1 prevented the expression of IRP1 and IRP2 in protein levels in MES23.5 cells. The protein levels of IRP1 (A) and IRP2 (B) were detected by Western blots. Results showed that both IRP1 and IRP2 protein levels in 6-OHDA-treated cells increased significantly compared with the control ($^{*}P < 0.05$, compared with the control). When pretreated with Rg1, the protein levels decreased significantly compared with 6-OHDA-treated cells and restored to the levels of the control ($^{\#}P < 0.05$, compared with 6-OHDA-treated cells). Data were presented as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

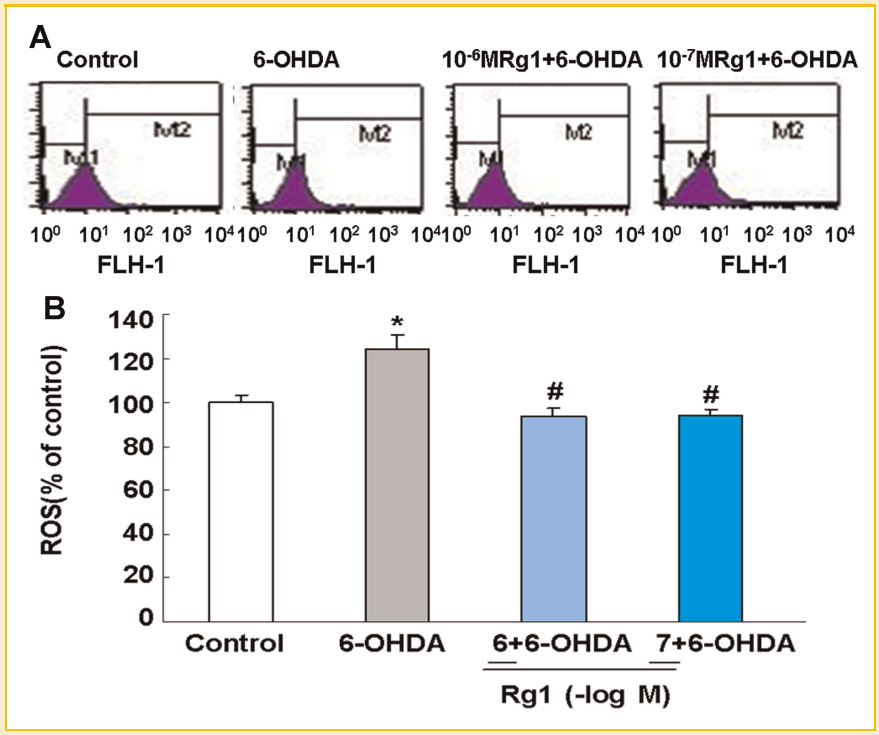

Fig. 6. Rg1 reduced 6-OHDA induced ROS generation. The levels of ROS in 6-OHDA-treated MES23.5 cells was significantly increased compared with control, while cells pretreated with Rg1 showed a relative low level of ROS. A: Representatives of the fluometric assay. B: Statistical analysis. Results were demonstrated as FL1-H (Fluorescence 1-histogram); setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellguest Software. %Gated for M2 was used to analysis the changes of the fluorescence. The control was set to 100% ($^{*}P < 0.05$, compared with the control; $^{\#}P < 0.05$, compared with 6-OHDA-treated cells). Data were presented as mean ± SEM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

This suggested that Rg1 could regulate the expression of IRP1 and IRP2 on mRNA and protein levels in MES23.5 cells so that regulating the expression of DMT1 + IRE. In addition to IRPs, there are also other factors that might be involved in the regulation of DMT1. It is postulated that transcription factors acting on putative AP-1, NF-kappaB-binding sites, or gamma-interferon responsive elements on the DMT1 promoter may also play a role in up-regulating the expression of the transporter [Huang et al., 2006]. However, the precise mechanisms of this regulation are still not fully elucidated and need further investigation.

The mechanisms underlying the regulation of Rg1 on the expression of IRPs are not clear. Accumulating evidence demonstrates that the generation of ROS might be involved in the regulation of the expression of IRPs and the subsequently regulate the iron-related proteins [Pantopoulos et al., 1996; Hanson and Leibold, 1999; Mladenka et al., 2006]. 6-OHDA could create oxidative stress through the production of H_2O_2 and other ROS, capable of destroying cellular structural and functional apparatus [Foley and Riederer, 2000; Hanrott et al., 2006]. This provided a hypothesis that Rg1 might suppress the expression of IRPs by inhibiting ROS generation. In this study, we demonstrated that Rg1 could decrease 6-OHDA-induced ROS production. This indicated that ginsenoside Rg1 might act as an antioxidant [Attele et al., 1999] and might protect neuronal damage through decreasing ROS-induced expression of IRPs and DMT1 + IRE and intracellular iron levels in 6-OHDA-treated cells.

In conclusion, we reported the neuroprotective effect of antioxidant Rg1 against iron toxicity in PD cell models. The mechanisms mediating this neuroprotective effect was due to its regulation of ROS-induced up-regulation of IRPs and DMT1 + IRE. This decreases the cellular iron levels and iron-induced degeneration of DA neurons. This provides new insight to understand the pharmacological effects of antioxidant on iron-induced degeneration of DA neurons.

ACKNOWLEDGMENTS

We thank Dr. Wei-dong Le to give us the MES23.5 cell line. This work was supported by grants from the National Natural Science Foundation of China (30870858, 30930036), and the Natural Science Fund of Shandong Province for Distinguished Young Scholars (JQ200807).

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