Ginsenoside Rd and ginsenoside Re offer neuroprotection in a novel model of Parkinson’s disease

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Abstract: Ginsenosides are the main active constituents of Panax ginseng. Ginsenoside Re is one of the major ginsenosides; whereas hydrolysis products such as Rd appear to have higher biological activity though are present in smaller amounts. Ginsenosides, from their early use in folk medicine to modern studies, appear to exert beneficial actions against aging and even neurodegenerative disorders. Parkinson’s disease is a progressive neurodegenerative movement disorder characterized by a profound loss of midbrain dopamine neurons in the substantia nigra pars compacta. Carbon tetrachloride (CCl4) exerts neurotoxic effects when present as an environmental pollutant. As a model compound it was used here to study the impact on primary nigrostriatal dopaminergic nerve cells and to investigate the neuroprotective potential of ginsenosides Rd and Re against this organic solvent. CCl4 (2.5 mM on day 12 in vitro for 48 h) significantly decreased the number of tyrosine hydroxylase (TH+) cells by 51% compared with untreated control cultures, reduced their neuritic lengths, and led to truncated degenerations of cell morphology. Ginsenosides Rd and Re (10 µM) strongly reduced cell loss and degeneration and significantly protected process lengths and numbers of neurites of TH+ cells. The anti-oxidative and anti-inflammatory potential of the cellular supernatant was lowered by CCl4 exposure. Inclusion of ginsenosides inhibited both oxidative stress and inflammation. Therefore the neuroprotective effects of ginsenosides at least partially depend on lowering oxidative stress and anti-inflammation.

Keywords: Ginsenoside Rd, ginsenoside Re, CCl4, dopaminergic neurons, Parkinson’s disease

Introduction

Parkinson’s disease (PD) is a progressive disorder characterized by the loss of nigrostriatal neurons. Although its symptoms and neuropathology have been well characterized, the exact etiology and the mechanisms underlying the degeneration of nigral dopaminergic neurons in PD are still unknown [1, 2]. For example, mitochondrial dysfunction, lipid peroxidation, increased accumulation of free iron, increased superoxide dismutase activity, and inflammation have all been implicated in nigral cell death. Thus, impaired energy metabolism, increased oxidative stress, and inflammation appear to be causative factors.

Carbon tetrachloride (CCl4), also called perchloromethane or tetrachloromethane, is a colorless, non-inflammable volatile liquid with a distinctive odor and is immiscible with water [2]. It is produced by chlorination of methane, ethane, propane, or propene. In its early applications this solvent was used in pharmaceutical preparations such as anaesthetics. However, it has long been known that inhalation of the vapor of this compound can depress central nervous system activity and cause degeneration of liver and kidneys through exerting a destructive and poisonous effect to both cells and organs, as is the case with many other well-known toxins.

After exposure to high levels of CCl4, the nervous system, including the brain, is affected. Though such exposure can be fatal, the immediate effects are usually signs of intoxication, including headache, dizziness, and sleepiness perhaps accompanied by nausea and vomiting.
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These effects usually disappear within a day or two after exposure stops. In severe cases, stupor or even coma can result, and permanent damage to nerve cells is possible.

Here we used primary dopaminergic cultures from embryonic mouse mesencephala to investigate in detail the potential molecular mechanisms underlying the degeneration of dopaminergic neurons induced by CCl₄. The direct toxicity of CCl₄ to dopaminergic neurons was assessed by immunocytochemical staining. Determination of lactate dehydrogenase (LDH) and lactate in the culture medium reflected the condition of the overall culture as well. We demonstrate that CCl₄ affected dopaminergic neurons in a dose-dependent manner. Ginsenosides or ginseng saponins as the active ingredients have antioxidant, anti-inflammatory, and immunostimulant properties, which raised speculations that these compounds could positively affect PD [3]. The potential neuroprotective effect of the two ginsenosides ginsenoside Rd and ginsenoside Re was investigated in these current studies.

Material and methods

Animals

Pregnant OF1/SPF mice at gestation day (GD) 14 were purchased from the Institute for Laboratory Zoology and Veterinary Genetics, Himberg (Austria). All animal care and surgical procedures were approved by the Animal Studies Committee of the University of Veterinary Medicine Vienna.

Reagents and drugs

Dulbecco’s Modified Eagle Medium (DMEM), fetal calf serum (FCS), CCl₄, L-glutamic acid (monosodium salt), poly-D-lysine, diaminobenzidine, and paraformaldehyde were purchased from Sigma (USA). Penicillin-streptomycin and anti-tyrosine hydroxylase (anti-TH antibody) were acquired from R&D Systems (USA). B-27 supplement (without antioxidants) was purchased from Gibco (UK). Vectastain ABC Elite Kit (Mouse IgG) was purchased from Vector Laboratories (USA). Sterile plastic ware was purchased from Nunc (USA).

Preparation of primary dopaminergic cell cultures

Pregnant animals were housed and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. On gestation day 14, pregnant mice were sacrificed under CO₂ anesthesia, their uteri were dissected, and the embryos were carefully removed and transferred to a Petri dish containing phosphate-buffered saline (PBS, pH 7.2). Under a Nikon stereoscope (SMZ-1B, 10 magnification), the brains were dissected, the ventral mesencephala excised, and primary cultures were prepared according to Radad et al. [1]. Briefly, after excision of ventral mesencephala, the meninges were carefully removed and tissues were mechanically cut into small pieces in PBS (pH 7.2) and subsequently triturated with a fire-polished Pasteur pipette in PBS (pH 7.2) and subsequently triturated with a fire-polished Pasteur pipette in PBS (pH 7.2) and subsequently triturated with a fire-polished Pasteur pipette in PBS (pH 7.2). Cultures were grown at 37°C in an atmosphere of 5% CO₂/5% air and 100% humidity. The culture medium was changed after 24 and 72 hours, and on the 5th day in vitro (DIV), serum was reduced to 5%, and 1% B 27 supplement was added to the medium. On the 6th DIV, the culture medium was changed to a serum-free medium containing 2% B 27 supplement.

Exposure of primary dopaminergic cell culture to CCl₄

CCl₄ was diluted to the desired final concentration with medium, containing HEPES buffer (25 mM), glucose (30 mM), penicillin and streptomycin (10 U/ml and 10 mg/ml, respectively) and glutamine (2 mM). For each treatment, fresh CCl₄ solution was used to compensate for evaporation by incubation. Cultures (10 DIV) were treated with 0.625, 1.25, or 2.5 mM CCl₄ for 48 h to investigate toxicity [2].

Identification of tyrosine hydroxylase immunoreactive (TH+)-cells

Anti-tyrosine hydroxylase (anti-TH) immunocytochemistry was performed using standard protocols. Cultures were washed three times with PBS (pH 7.2) for 5 min. Except for the incubation with anti-TH antibody, the staining procedure was performed at room temperature. Briefly, cells were rinsed in PBS (pH 7.2) and fixed for 30 min with 3.7% formalin solution,
permeabilized with Triton-X 100 for 30 min, and unspecific binding sites were blocked by 5% horse serum for 90 min. Cells were incubated with mouse anti-TH antibody overnight at 4°C. The secondary antibody and the avidin-biotin horseradish peroxidase complex were added to the cells, permitting 90 min for incubation in each case (Vectastain ABC-kit). The immunoreaction was visualized by dianaminobenzidine (1.4 mM in PBS; 3.3 mM H₂O₂) under optical control (Nikon inverted microscope, 10x magnification). TH+ cells were counted in 40 adjoining fields (2.8 mm² per field). For statistical analysis, the values of the 10 fields with the highest cell numbers were calculated. Data were obtained from three different cultures (4 wells for each treatment condition in each culture).

**Lactate dehydrogenase (LDH) release assay**

Neuronal cell injury was quantitatively assessed by measuring the activity of LDH released from damaged cells into the culture medium. The reaction was initiated by mixing 0.2 ml of cell-free supernatant (diluted 1:1 with aqua dest.) with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate (final concentrations 11 and 37 μM, respectively, in 350 μM potassium phosphate buffer pH 7.2) in a final volume of 0.5 ml. The decrease of NADH was spectrophotometrically (NOVASPEC II) monitored. Reagent blanks were subtracted. LDH activity was calculated from the slope of the decrease in optical density at 350 nm; LDH release is proportional to the number of damaged or destroyed neurons.

**Fluorescence microscopy**

The detection of oxidative stress: Propidium iodide (PI) staining was performed in the primary dopaminergic cell cultures on the 11th DIV. PI (final concentration 2 mM in colorless DMEM) was added to the cultures and incubated at 37°C for 10 min. Superoxide radical detection was performed using dihydroethidium (DHE; hydroethidine). DHE (10 μM) was added to cultured cells at 37°C for 30 min. DHE is oxidized in the presence of superoxide radicals to ethidium, which intercalates into DNA and emits an intense red fluorescence. After incubation with each fluorescent dye, cultured cells were washed with colorless medium, and 10 photos were taken by a Coolpix 990 digital camera (10x magnification; Nikon, Japan) attached to an inverted microscope (Nikon, Japan) with epifluorescence equipment using TRITC (G-2A) and FITC (B-2A) filters for DHE and PI, respectively. Color intensities for each photo were analyzed by Adobe Photoshop software. The fluorescence intensity, which directly correlates with the superoxide radical concentration, was measured with the histogram modus in regions of interest (1.68 mm²/field).

The detection of inflammation: Nitric oxide (NO) detection was performed using 4-amino-5-methylamino-2,7-difluoro: fluoresceindiacetate (DAF-FM diacetate). DAF-FM diacetate (10 μM) was incubated with cultured cells at 37°C for 90 min. DAF-FM diacetate enters cells, where it is deacetylated by esterases to the nonfluorescent DAF-FM, which then reacts with NO to form fluorescent benzotriazole to produce a diffuse staining. After incubation with each fluorescence dye, cultured cells were washed with colorless medium and 10 photos were taken with a Coolpix 990 digital camera (at 10x magnification; Nikon, Japan) attached to an inverted microscope (Nikon, Japan) with epifluorescence equipment using TRITC (G-2A) and FITC (B-2A) filters for PI and DAF-FM, respectively. The fluorescence intensity directly correlates with NO concentration. Color intensities for each photo were analyzed by Adobe Photoshop software. The averaged color intensity was measured with the histogram modus in regions of interest (1.68 mm²/field).

**Statistics**

Data from counting cells were statistically evaluated. Statistically significant differences were determined using the Kruskal-Wallis (H)-test followed by the Chi²-test. Differences with p < 0.05 were regarded as statistically significant.

**Results**

**Effects of CCl₄ on the survival of mesencephalic dopaminergic cells**

Dopaminergic neurons in primary culture undergo degeneration when exposed to CCl₄. Cultures were treated for 10 days with different concentrations of CCl₄ (0.625, 1.25, and 2.5 mM) from the 10th DIV. Numbers of dopaminergic cells decreased in a dose-dependent manner (Figure 1A). Since 2.5 mM CCl₄ induced significant dopaminergic cell death, we used
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2.5 mM for further investigations. Changes in morphology were manifested in losses of dendritic processes and deterioration of cell shape in primary dopaminergic neurons as a result of CCl₄ exposure (Figure 1B, upper left panel) compared with those from untreated control primary dopaminergic neurons (Figure 1B, upper right panel).

Ginsenoside Rd protected dopaminergic neurons against carbon tetrachloride-induced neurotoxicity

Significant dopaminergic cell loss underlies the primary pathology of PD. We tested the effects of ginsenoside Rd on CCl₄-induced dopaminergic cell death. Mesencephalic cultures were...
pretreated with ginsenoside Rd (1-10 µM) before including CCl₄ (2.5 mM). After the 12-day treatment period, TH⁺-positive neurons were quantitatively counted. The number of TH⁺ neurons in control neurons were compared to CCl₄ treated cells with or without Ginsenosides Rd or Ginsenoside Re (approx. 600 cells per well). Data are given as mean values ± SEM of 4 individual determinations.

Ginsenoside Re protected dopaminergic neurons against carbon tetrachloride-induced neurotoxicity

We tested the effects of ginsenoside Re on CCl₄-induced dopaminergic cell death. Mesencephalic dopaminergic cultures (DIV 12) were pretreated with ginsenoside Re (1-10 µM) for 2 hours and then treated with 2.5 mM CCl₄ for 48 hours. TH⁺-immunostained neurons were counted. As shown in Figure 1B (lower right panel) and Figure 2A, Ginsenoside Re (1-10 µM) exerted a significant neuroprotective effect on dopaminergic neurons compared to other cultures treated with CCl₄.

Ginsenoside Rd decreased LDH activity

We previously detected cytotoxicity in a variety of neuronal cell cultures by LDH assay [4, 5, 6] since the release of LDH serves as an indicator of general cytotoxicity. Addition of 2.5 mM CCl₄ to mesencephalic dopaminergic cell cultures on the 12th DIV for 48 hours caused considerable release of LDH. LDH activity was increased 1.49-fold compared with control cells (Figure 3A). We found that the administration of ginsenoside Rd reduced LDH activity in a dose-dependent manner. In CCl₄-treated cells, ginsenoside Rd (1 and 5 µM) reduced LDH activity (3% and 3.5%, respectively), while higher dose of ginsenosides Rd (10 µM) significantly reduced LDH activity 18.5%. Thus our data suggest that ginsenoside Rd inhibits CCl₄-induced neurotoxicity in mesencephalic dopaminergic cell cultures.

Ginsenoside Re decreased LDH activity

Addition of CCl₄ (2.5 mM) to mesencephalic dopaminergic cell cultures on the 12th DIV for 48 hours caused significant release of LDH into
the culture medium. We demonstrate that LDH activity was increased 1.49-fold compared to controls (Figure 3B). Same as ginsenoside Rd did, the administration of ginsenoside Re re-
duced LDH activity in a dose-dependent man-
er as well. In CCl\textsubscript{4}-treated cells, ginsenoside Re (1 µM) reduced LDH activities by 3.4%, while higher doses of ginsenoside Re (5 µM and 10 µM) significantly reduced LDH activity (12.6% and 20.9%, respectively). Our data therefore suggest that ginsenoside Re inhibits CCl\textsubscript{4}-induced neurotoxicity in mesencephalic dopaminergic neurons.

**Ginsenoside Rd decreased superoxide formation**

The superoxide indicator dihydroethidium exhibits blue-fluorescence in the cytosol until it is oxidized, when it intercalates with DNA in the primary dopaminergic cells and produces a bright fluorescent red staining in the nucleus. Exposure to 2.5 mM CCl\textsubscript{4} for 48 h on the 10th DIV increased superoxide radicals 1.72-fold compared to untreated controls (Figure 4A). When CCl\textsubscript{4}-treated cultured primary dopaminergic cells were treated with ginsenoside Rd, superoxide radical formation was significantly reduced. Ginsenoside Rd reduced superoxide radical formation in a dose-dependent manner, i.e., 1, 5, and 10 µM of ginsenoside Rd reduced superoxide radical formation by 12.2%, 13.9%, and 18.6%, respectively, compared with cultures treated with CCl\textsubscript{4} alone (Figure 4A).

**Ginsenoside Re decreased superoxide formation**

Control primary dopaminergic cultures generally exhibited low fluorescence intensity, while CCl\textsubscript{4}-treated cultures showed bright red fluorescence. Treatment of cultures with 2.5 mM CCl\textsubscript{4} for 48 h on the 10th DIV increased superoxide radicals 1.72-fold compared to untreated controls (Figure 4B). When cultured cells were pre-
treated with ginsenoside Re, superoxide radical formation as a result of CCl₄ exposure was significantly reduced. At 1, 5, and 10 μM, ginsenoside Re reduced superoxide radical formation dose-dependently by 8.7%, 12.2%, and 15.1%, respectively, compared to CCl₄-treated cells. Ginsenoside Rd and ginsenoside Re decreased nitric oxide DAF-FM produced diffuse staining. Administration of 2.5 mM CCl₄ led to a 1.86-fold rise in nitric oxide in embryonic mesencephalic cultures within 48 h on the 10th DIV. Cultures were treated with ginsenoside Rd at different concentrations for 48 hours from the 10th DIV. Ginsenoside Re (1 μM) decreased nitric oxide by 8% compared with CCl₄ alone (Figure 5A), while 5 and 10 μM reduced nitric oxide 14 and 16.6%, respectively. Similar with ginsenoside Rd did, when cultured cells were pretreated with ginsenoside Re, nitric oxide production as a result of CCl₄ exposure was significantly reduced as well. At 1, 5, and 10 μM, ginsenoside Re reduced superoxide radical formation dose-dependently by 8, 15, and 19.8%, respectively (Figure 5B). Our data suggest that the protective effect of ginsenoside Rd and ginsenoside Re in dopaminergic neuronal cell death are summarized.

Discussion

Since PD has a high prevalence, a high morbidity rate, and a chronic long-lasting disease process, it is becoming an issue of widespread concern and associated with serious medical and social problems in the field of senior health. Although we and other researchers have tested many drugs [2, 3, 7, 8], stem cells [9, 10], and a variety of other treatments against PD, so far there is no therapy that can effectively reverse, block, or even slow the degeneration of neu-
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Ginsenosides, the bioactive components of ginseng, derive from the triterpene dammarane and can be classified into two classes: the protopanaxadiol derivatives, consisting mainly of Rb1, Rb2, Rc, and Rd; and protopanaxatriol derivatives consisting mainly of Re, Rf, Rg1, and Rg2 [11]. Recent studies in neuronal cells demonstrate that ginsenosides, the active ingredients of Panax ginseng roots, exert protective effects on neuronal cells [12].

In our present study, ginsenoside Rd treatment at concentrations of 5 and 10 µM significantly preserved the total number of TH+ neurons in primary dopaminergic neurons compared with CCl4-treated cultures (Figure 1). This result is in agreement with Jiang et al. [13], who reported the neuroprotective effects of ginsenoside Rd on survival of dopaminergic cells. Furthermore, Choi et al. [14], also reported that ginsenoside Rd inhibited kainic acid-induced neurotoxicity in mice by a dose-dependent manner.

Ginsenoside Re at concentrations of 5 and 10 µM significantly preserved neurite lengths and cell numbers of TH+ neurons compared with CCl4-damaged cultures. Neuroprotection by ginsenoside Re has also been described in a few investigations using other neurodegeneration models. For instance, experiments using an in vitro model of cellular injury induced by amyloid also demonstrated the neuroprotective effect of ginsenoside Re and found that it is capable of protecting PC12 cells from the damage induced by serum-free medium [15]. Xu et al. [16] found that ginsenoside Re showed protection against MPTP-induced apoptosis in the PD model of mouse nigral neurons. In addition, ginsenosides Re from Panax ginseng induce neuroprotection [17]. López et al. [18] reported that ginsenoside Rb1, Rb2, Re, and Rg1 were also effective in reducing astrocyte death.

Our data that ginsenosides inhibiting the decrease of antioxidant enzymes (Figure 6) provide additional support for the contention that ginsenosides exert protective effects due to their antioxidant capability. Ginsenosides Re from Panax ginseng induce neuroprotection mainly through activation of antioxidant enzymes [17]. Ginsenoside Rb1, Rb2, Rd, Re, and Rg1 decreased ROS formation, with ginsenoside Re being the most active [18].

Our present studies also confirm that ginsenoside Rd and ginsenoside Re reduced nitric oxide, therefore showing their anti-inflammatory abilities in primary dopaminergic neurons upon CCl4 insult (Figure 6). Our finding is consistent with the previous report that ginsenoside Rd exerted a significant neuroprotective effect on dopaminergic neurons compared to cultures treated with lipopolysaccharide (LPS) [19]. Studies indicated that the neuroprotective effect of ginsenoside Rd against LPS toxicity relates to anti-inflammatory mechanisms [19]. Xu et al. [16] found that ginsenoside Re showed protection against MPTP-induced cell death in the PD model of mouse nigral neurons, and that this effect may be attributable to downregulating the expression of iNOS protein [16].

The neuroprotection of ginsenosides also involved other anti-apoptosis and energy metabolism mechanisms. Ginsenoside Re provided protection against MPTP-induced apoptosis in the PD model of mouse nigral neurons, and that this effect may be attributable to upregulating the expression of Bcl-2 protein and downregulating the expression of Bax protein, and inhibiting the activation of caspase-3 [16]. Furthermore, the neuroprotective effects of ginsenoside Rd on survival of dopaminergic cells has been reported to be mediated through improving energy metabolism and preserving the structural integrity of neurons [13]. Moreover CCl4 is a well known lipid-soluble toxic agent that is activated in the liver by the cytochrome P-450 system. The toxicity of CCl4 results from its reductive dehalogenation by the cytochrome P-450 enzyme system into a trichloromethyl free radical, which is believed to initiate a series of biochemical events that are ultimately expressed as liver cell necrosis. The trichloromethyl radicals can form covalent adducts with lipids and proteins, which readily interact with molecular oxygen to form the trichloromethyl peroxyl radical [20]. Both radicals can attack proteins and lipids or abstract hydrogen atoms from an unsaturated lipid, leading to membrane lipid peroxidation, cellular dysfunction, and eventually to cell death. However there are no reports concerning CCl4 neurotoxicity in cellular models of PD. Our report shows...
that CCl4 is a novel inducer for primary dopaminergic neuronal cell death, therefore CCl4-mediated primary dopaminergic neuronal cells may provide a new screening tool for the drug discovery against PD and the investigation of pathogenesis of PD. Furthermore, our study for the first time demonstrates the neuroprotective potential of ginsenosides Re and Rd against CCl4-mediated primary dopaminergic neuronal cell death.

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Disclosure of conflict of interest

None.

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