Neuroprotective Effect of Protein Kinase Cδ Inhibitor Rottlerin in Cell Culture and Animal Models of Parkinson’s Disease

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ABSTRACT

Recent studies from our laboratory demonstrated that the protein kinase C (PKC) δ isoform is an oxidative stress-sensitive kinase and a key mediator of apoptotic cell death in Parkinson’s Disease (PD) models (Eur J Neurosci 18:1387–1401, 2003; Mol Cell Neurosci 25:406–421, 2004). We showed that native PKCδ is proteolytically activated by caspase-3 and that suppression of PKCδ by dominant-negative mutant or small interfering RNA against the kinase can effectively block apoptotic cell death in cellular models of PD. In an attempt to translate the mechanistic studies to a neuroprotective strategy targeting PKCδ, we systematically characterized the neuroprotective effect of a PKCδ inhibitor, rottlerin, in 1-methyl-4-phenylpyridinium (MPP+)–treated primary mesencephalic neuronal cultures as well as in an 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of PD. Rottlerin treatment in primary mesencephalic cultures significantly attenuated MPP+–induced tyrosine hydroxylase (TH)-positive neuronal cell and neurite loss. Administration of rottlerin, either intraperitoneally or orally, to C57 black mice showed significant protection against MPTP-induced locomotor deficits and striatal depletion of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid. Notably, rottlerin post-treatment was effective even when MPTP-induced depletion of dopamine and its metabolites was greater than 60%, demonstrating its neurorescue potential. Furthermore, the dose of rottlerin used in neuroprotective studies effectively attenuated the MPTP-induced PKCδ kinase activity. Importantly, stereological analysis of nigral neurons revealed rottlerin treatment significantly protected against MPTP-induced TH-positive neuronal loss in the substantia nigra compacta. Collectively, our findings demonstrate the neuroprotective effect of rottlerin in both cell culture and preclinical animal models of PD, and they suggest that pharmacological modulation of PKCδ may offer a novel therapeutic strategy for treatment of PD.
teolytic activation of PKCδ, a member of the novel PKC isoform family, plays a critical role in oxidative stress-induced dopaminergic cell death in cell culture models of PD (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004). Proteolytic cleavage of PKCδ (74 kDa) by caspase-3 results in a 41-kDa catalytic subunit and a 38-kDa regulatory subunit, leading to a persistent activation of the kinase (Kaul et al., 2003; Yang et al., 2004). Blockage of proteolytic activation of PKCδ by overexpression of the kinase-dominant-negative PKCδ mutant, cleavage-resistant PKCδ mutant, or siRNA directed against PKCδ almost completely prevented the dopaminergic cell death (Kaul et al., 2003; Kitazawa et al., 2003; Yang et al., 2004; Latchoumycandane et al., 2005). Therefore, in the present study, we examined the neuroprotective efficacy of the PKCδ inhibitor rottlerin (a.k.a. mallotoxin) in both primary cell culture and animal models. Herein, we report that rottlerin not only protects against MPP+/H9254 induced degeneration of tyrosine hydroxylase (TH)-positive neurons in primary mesencephalic culture models but also, most importantly, it is clearly neuroprotective in an MPTP animal model of Parkinson’s disease.

Materials and Methods

Chemicals and Biological Reagents. Rottlerin, 1-methyl-4-phenylpyridinium, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, protease cocktail, ATP, protein A-Sepharose, protein G-Sepharose, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); mouse TH antibody was purchased from Chemicon International (Temecula, CA); rabbit PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and anti-rabbit and anti-mouse secondary antibodies and the enhanced chemiluminescence kit were purchased from GE Healthcare (Piscataway, NJ). [γ-32P]ATP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). The Bradford protein assay kit was purchased from Bio-Rad (Hercules, CA). Neurobasal medium, B27 supplement, l-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD).

Mesencephalic Primary Neuron Cultures and Treatment. Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon of gestational 16- to 18-day-old mice embryos as described in our previous publication (Yang et al., 2004). We used E16 to 18 mouse embryos for preparation of primary mesencephalic cultures because the number of TH+ neurons in ventral mesencephalon of E16 to 18 embryos is almost twice the number in E14 embryos (Piszman et al., 1991). In addition, the DA level, DA uptake sites, and striatal innervation were clearly greater in E16 to 18 than in E14 embryos. Mesencephalic tissues from E16 to 18 mouse embryos were dissected and maintained in ice-cold Ca2+-free Hanks’ balanced salt solution and then dissociated in Hanks’ balanced salt solution containing trypsin-0.25% EDTA for 20 min at 37°C. The dissociated cells were then plated at equal density (0.5 × 106 cells) on 12-mm coverslips precoated with 1 mg/ml poly-d-lysine. Cultures were maintained in a chemically defined medium consisting of neurobasal medium fortified with B-27 supplements, 500 μM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified CO2 incubator (5% CO2 and 37°C) for 24 h, and they were treated with cytosine arabinoside at 10 μM for 24 h to inhibit glial cell proliferation. Half of the medium was given to controls. For the rottlerin pretreatment study, rottlerin and DMSO administrations were started 24 h before the injections of MPTP, and they were continued for 5 days. In the subchronic MPTP administration regimens, MPTP in PBS was injected intraperitoneally at doses of 30 mg/kg, once per day for 5 days. Mice were sacrificed 7 days after the last MPTP injection, and the striata were dissected for catecholamine analysis. The mesencephalon were fixed in 4% paraformaldehyde for TH immunostaining. For the rottlerin post-treatment study, mice were treated with 20 mg/kg i.p. MPTP once a day for 3 days, and then rottlerin was administered at 20 mg/kg p.o. once a day for an additional 3 days. Mice were sacrificed 7 days after the MPTP injections, and the striata were dissected for neurochemical analysis.

Immunocytochemistry. After treatment, the primary mesencephalic neurons were fixed with 4% paraformaldehyde and processed for immunocytochemical staining. First, nonspecific sites were blocked with 5% normal goat serum containing 0.4% bovine serum albumin and 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 20 min. Cells were then incubated with antibody directed against TH (1:500 dilution) at 4°C overnight, followed by incubation with Cy3-conjugated (red; 1:1000) secondary antibody for 1 h at room temperature. Secondary antibody treatments were followed by incubation with 10 μg/ml Hoechst 33342 for 3 min at room temperature to stain the nucleus. Cultures from each batch of embryos were processed under identical conditions, including both primary and secondary antibody concentrations and incubation times. The results were compared between the control group and treatment groups. The coverslips containing stained cells were washed with PBS, mounted on a slide, and viewed under a Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan); images were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Quantification of TH+ Cell Count and Neuronal Processes. MetaMorph software, version 5.0 (Molecular Devices, Sunnyvale, CA) was used for measurement of TH+ cells and neuronal processes in primary neurons from each coverslip. For measurement of TH cell count, the images were first thresholded, and then neuronal count and volume were measured using the Integrated Morphometry Analysis function. The data were logged to an Excel spreadsheet (Microsoft, Redmond, WA) and analyzed. For measurement of neuronal processes, the lengths of the processes were marked by applying an ellipse of length and region measurement function in the Integrated Morphometry Analysis. The data were exported to an Excel spreadsheet and analyzed. TH+ neurons and their processes were counted in at least six individual cultures for each treatment. This is a modified version of a method recently used for quantification of neuronal processes (Yang et al., 2004).

Animal Studies and Rottlerin Treatment. Six- to 8-week-old 26/CD7/Bl mice weighing 25 to 30 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Committee on Animal Care at Iowa State University (Ames, IA). Rottlerin was dissolved in 1% dimethyl sulfoxide (DMSO), and it was administered either intraperitoneally or orally at various doses ranging from 3 to 20 mg/kg. An equal volume of DMSO was given to controls. For the rottlerin pretreatment study, rottlerin and DMSO administrations were started 24 h before the injections of MPTP, and they were continued for 5 days. In the subchronic MPTP administration regimens, MPTP in PBS was injected intraperitoneally at doses of 30 mg/kg, once per day for 5 days. Mice were sacrificed 7 days after the last MPTP injection, and the striata were dissected for catecholamine analysis. The mesencephalon were fixed in 4% paraformaldehyde for TH immunostaining. For the rottlerin post-treatment study, mice were treated with 20 mg/kg i.p. MPTP once a day for 3 days, and then rottlerin was administered at 20 mg/kg p.o. once a day for an additional 3 days. Mice were sacrificed 7 days after the MPTP injections, and the striata were dissected for neurochemical analysis.

Immunohistological and Stereological Analysis of Nigral Sections. In brief, brains were harvested following cervical dislocation, postfixed in paraformaldehyde, and used for TH immunolabeling and stereological analysis as described previously (Thiruchelvam et al., 2004). Fixed brains were cut into 30-μm sections, and the sections were collected in cryoprotectant. Sections were rinsed in PBS at room temperature before immunostaining, and then they were incubated with anti-TH antibody. The total number of TH+ and Nissl-stained neurons in the SNc were counted using an optical fractionator with the criteria described previously by others (Thiruchelvam et al., 2004). After delineation of the region at low magnification (4× objective), every fourth section from the entire substantia nigra was sampled at higher magnification (100× objective) using a Stereoinator (Microbrightfield, Burlington, VT).
PKCδ Kinase Assay. PKCδ enzymatic activity was assayed using an immunoprecipitation kinase assay as described previously (Anantharam et al., 2002). In brief, substantia nigra brain tissue from rottlerin-, MPTP-, and MPTP + rottlerin-treated and control-treated mice was washed once with PBS, and then tissue was resuspended in 1 ml of PKC lysis buffer (25 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM NaF, and 4 μg/ml each of aprotinin and leupeptin). The lysates were cooled on ice for 30 min, and then they were centrifuged at 15,000g for 5 min. The supernatants were collected as cytosolic fractions.

Protein concentration was determined using a Bradford assay (Bradford, 1976). Cytosolic protein (0.25–0.5 mg) was immunoprecipitated overnight at 4°C using 2 μg of anti-PKCδ antibody. The immunoprecipitates were then incubated with protein A-Sepharose (Sigma-Aldrich) for 1 h at 4°C. The protein A-bound antigen-antibody complexes were then washed three times with 2× kinase buffer (40 mM Tris, pH 7.4, 20 mM MgCl₂, 20 μM ATP, and 2.5 mM CaCl₂), and then they were resuspended in 20 μl of 2× kinase buffer.

The reaction was started by adding 20 μl of reaction buffer containing 0.4 mg of histone H1, 50 μg/ml phosphatidylserine, 4.1 μM dioleoylglycerol, and 5 μCi of [γ-32P]ATP at 3000 Ci/mM to the immunoprecipitated samples, and the sample was incubated for 10 min at 30°C. SDS gel-loading buffer (2×) was added to terminate the reaction, the samples were boiled for 5 min, and the products were separated on a 12.5% SDS-polyacrylamide gel electrophoresis gel. The H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model; Bio-Rad), and the bands were quantified using Quantity One 4.2.0 software (Bio-Rad).

HPLC Analysis of Neurotransmitters. DA and DOPAC levels in brain striatal tissues were determined by high-performance liquid chromatography (HPLC) with electrochemical detection; samples were prepared as described previously (Kitazawa et al., 2001). In brief, neurotransmitters were extracted from samples using an antioxidant extraction solution (0.1 M perchloric acid containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₃). The extracts were filtered in 0.22-μm spin tubes, and 20 μl of the samples was loaded for analysis. DA and DOPAC were separated isocratically by a reversed-phase column with a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an automatic sampler equipped with refrigerated temperature control (model 542; ESA Inc.) was used for these experiments. The electrochemical detection system consisted of a Coulomet model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). The standard stock solution of catecholamines was prepared at 1 mg/ml in antioxidant solution, and then it was further diluted to a final working concentration of 50 pg/μl before injection. The data acquisition and analysis were performed using the EZStart HPLC Software (ESA Inc.). The DA and DOPAC levels were quantified as nanograms per milligram of protein.

Western Blot. Brain lysates containing equal amounts of protein were loaded in each lane and separated on a 10 to 12% SDS-polyacrylamide gel electrophoresis gel as described previously (Kaul et al., 2003). After the separation, proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with 5% nonfat dry milk powder. The membranes were then treated with primary antibodies directed against TH (mouse monoclonal; 1:1000). The primary antibody treatments were followed by treatment with secondary horseradish peroxidase-conjugated antimuscle IgG (1:2000) for 1 h at room temperature. Secondary antibody-bound proteins were detected using an enhanced chemiluminescence kit (GE Healthcare). To confirm equal protein loading, blots were reprobed with a β-actin antibody (1:5000 dilution). Western blot images were captured with a Kodak 2000 MM imaging system (Eastman Kodak, Rochester, NY), and data were analyzed using one-dimensional Kodak Imaging Analysis software (Eastman Kodak). The densitometric values of TH bands were normalized to the β-actin band.

Locomotor Activity. Behavioral data were collected using VersaMax animal activity monitors (model RXYZCM-16; Accuscan Instruments Inc., Columbus, OH). Each chamber was 40 × 40 × 30.5 cm, made of clear Plexiglas, and covered with a Plexiglas lid with holes for ventilation. Infrared monitoring sensors were located every 2.54 cm along the perimeter (16 infrared beams along each side) and 2.5 cm above the floor. Two additional sets of 16 sensors were located 8.0 cm above the floor on opposite sides. Data were collected and analyzed by a VersaMax analyzer (model CDA-8; Accuscan Instruments Inc.), which in turn sent information to a computer where it was stored for future analyses. Locomotor activity was presented as horizontal movement and vertical movement. All data are expressed as percentage of the vehicle-treated control group (mean ± S.E.M.; n = 6) and were obtained 1 day after MPTP or vehicle treatment in a 20-min test session.

Data Analysis. Data analysis was performed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Raw data were first analyzed using one-way analysis of variance and then Bonferroni’s post-test was performed to compare all treatment groups. Differences with p < 0.05 were considered significant.

Results

Rottlerin Rescues TH⁺ Neuronal Loss from MPP⁺ in Primary Mesencephalic Cultures. We first examined whether the PKCδ inhibitor rottlerin (Fig. 1) can rescue TH⁺ neurons from MPP⁺ toxicity in primary mesencephalic cultures. Primary mesencephalic cultures were isolated from E16 to 18 C57 black mice embryos, and neurons were exposed to 10 μM MPP⁺ in the presence or absence of 0.3 and 1 μM rottlerin for 48 h. After treatment, primary neurons were processed for TH immunohistochemistry. As shown in Fig. 2A, MPP⁺ treatment induced an approximately 90% loss of both TH cell count and neurite processes. Quantitative analysis of TH⁺ cell counts showed that 0.3 and 1 μM rottlerin treatment significantly protected against MPP⁺ neurotoxicity. Likewise, average lengths of TH⁺ neuronal processes in MPP⁺ plus rottlerin-treated primary neurons were significantly longer than the processes of neurons treated only with MPP⁺ (Fig. 2B). These data suggest that the PKCδ inhibitor rottlerin possesses neuroprotective properties in the MPP⁺-induced dopaminergic degenerative model.

PKCδ Kinase Activity Is Significantly Suppressed in Rottlerin-Treated Animals. We previously showed that pretreatment with rottlerin significantly attenuated oxidative stress-induced increases in PKCδ kinase activity in an immortalized mesencephalic dopaminergic neuronal cell line (Kaul et al., 2003). We also observed a high expression of PKCδ in TH-positive nigral neurons (unpublished observation). In this experiment, we examined whether rottlerin
effectively inhibits PKC activity in mouse substantia nigra. Rottlerin was administered at doses of 3 to 20 mg/kg. As shown in Fig. 3, PKC activity was significantly suppressed in the substantia nigra of rottlerin-treated animals in a dose-dependent manner compared with vehicle-treated control animals. Densitometric analysis of phosphorylated histone H1 bands revealed 48, 37, and 29% decreases in protein kinase activity in SN of mice treated with 20, 10, and 7 mg of rottlerin, respectively. These results indicate that rottlerin significantly inhibits PKC activity, as observed in our previous cell culture experiments (Kaul et al., 2003).

Rottlerin Attenuates Striatal Dopamine Depletion Induced by MPTP Treatment. After characterizing the neuroprotective effect of rottlerin in cell culture models of PD as well as verifying the ability of rottlerin to suppress PKC activity in the brain, we examined the neuroprotective efficacy of rottlerin in an MPTP-induced mouse model of PD. To determine the optimal route of rottlerin treatment, we chose the intraperitoneal route for low doses of rottlerin (3 and 7 mg/kg) and the oral route for high doses (10 and 20 mg/kg). Animals were administered rottlerin (3–20 mg/kg body weight) 24 h before MPTP administration. Animals were then cotreated with rottlerin and MPTP (30 mg/kg i.p.) daily for 5 days. Saline- and vehicle-injected animals were used as controls. To assess the protective effect of rottlerin, we first examined whether rottlerin could block MPTP-induced loss of striatal dopamine and its metabolites. As shown in Fig. 4, MPTP treatment induced loss of dopamine (>94%) (A),...
DOPAC (>95%) (B), and HVA (>95%) (C) in mouse striatum. The dopamine levels were determined to be 187.5 ± 7.4, 14.5 ± 3.8, and 112.8 ± 25.3 ng/mg protein in control-, MPTP-, and MPTP + 20 mg/kg rottlerin-treated animals, respectively. Pretreatment with 20 mg/kg rottlerin afforded nearly a 50% protection against MPTP-induced striatal dopamine loss. Similar results were found for DOPAC and HVA (Fig. 4). Furthermore, the metabolite to amine ratio showed an increase with MPTP-induced nigral damage, which is consistent with existing findings. Rottlerin was measured in nigral tissue at 1125 pg/mg tissue, indicating that rottlerin effectively reaches the target tissue. Collectively, these data suggest that rottlerin treatment could afford protection against MPTP-induced striatal dopamine and dopamine metabolite loss in animal PD models.

**Rottlerin Inhibits PKCα Kinase Activity in MPTP-Treated Animals.** In a previous study, we showed that PKCα activity was increased in MPP⁺-treated N27 dopaminergic cells as a result of proteolytic kinase activation (Kaul et al., 2003). Therefore, we examined whether 20 mg/kg rottlerin, the most effective dose against MPTP-induced dopamine depletion, suppressed MPTP-induced increases in PKCα kinase activity in nigral tissue. In Fig. 5, MPTP-treated mice showed nearly a 2-fold increase in PKCα activity compared with control mice, and rottlerin treatment almost completely suppressed PKCα kinase activity induced by MPTP treatment. These results suggest that rottlerin protects against PKCα activation.

**Rottlerin Attenuates TH Cell Loss Induced by MPTP Toxicity in SNc.** Because rottlerin effectively attenuated MPTP-induced loss of dopamine and its metabolites in the striatum, we next examined whether rottlerin could protect the TH⁺ neurons from MPTP-induced toxicity in the SNc (Fig. 6A). Stereological analysis of TH⁺ cell count revealed that MPTP treatment caused a 40% loss of TH⁺ neurons in the SNc compared with saline-treated animals. However, MPTP induced only a 13% loss of TH⁺ neurons in animals treated with 20 mg/kg p.o. rottlerin (Fig. 6B). Data analysis revealed that rottlerin treatment afforded a 37% protection against MPTP. In addition, there was a noticeable loss of TH⁺ fiber density in MPTP-treated animals, whereas fiber density in the rottlerin-treated animals was close to that of the vehicle-treated group (Fig. 6A).

**Rottlerin Prevents MPTP-Induced Reduction of TH Protein in SNc.** Based on our finding that rottlerin counteracted the MPTP-induced dopamine and TH⁺ neuronal loss, we further confirmed nigral TH protein levels by Western blot. MPTP treatment greatly reduced TH protein levels in mouse SN (Fig. 7), which may have resulted from the loss
of TH⁺ neurons in this region. No significant change in β-actin level was noted among the different groups. Normalized densitometric results revealed that rottlerin treatment restored the TH levels in MPTP-treated animals to those of control animals, further confirming the protective effect of rottlerin against TH-positive neuronal loss.

**Rottlerin Attenuates MPTP-Induced Motor Deficits.** With evidence suggesting that rottlerin protects against MPTP-induced dopamine and TH neuronal loss, we next determined whether rottlerin treatment could also afford protection against MPTP-induced motor deficits. We compared the motor activity of vehicle, MPTP, and MPTP plus rottlerin-treated animals, using a VersaMax computerized activity monitoring system (Accuscan, Columbus, OH). This system uses infrared sensors to measure repetitive movements both in the horizontal and vertical planes in real time, and it provides color-coded output. Representative activity maps of the animals are presented in Fig. 8A. The cumulative horizontal and vertical activities are shown in Fig. 8B. All data are expressed as percentage of the vehicle-treated group, and they were obtained 1 to 2 h before sacrificing the animals for neurochemical and histological studies. Statistical analyses of both raw data and percentage of control showed significant differences. The data indicate that both the vertical and horizontal motor activity were significantly reduced (>45%) in MPTP-treated animals compared with the vehicle-treated group. Administration of rottlerin partially restored both vertical and horizontal motor activity of MPTP-treated animals to the levels observed in control animals. These results demonstrate rottlerin treatment attenuates MPTP-induced locomotor deficits in mice.

**Rottlerin Post-Treatment Rescues Striatal Dopamine Depletion Induced by MPTP Treatment.** To determine whether rottlerin post-treatment can protect against MPTP-induced dopaminergic toxicity, we created a PD model that shows more than 60% of striatal dopamine depletion by pretreating the mice with 20 mg/kg i.p. MPTP for 3 days. After the MPTP treatment, animals were given 20 mg/kg rottlerin for 3 days. As shown in Fig. 9, post-treatment with rottlerin significantly attenuated the loss of dopamine induced by MPTP pretreatment. Post-treatment with 20 mg/kg rottlerin afforded nearly a 33% protection against MPTP pretreatment-induced striatal dopamine loss, indicating the effectiveness of the kinase inhibitor against the ongoing neurodegenerative process. Similar results were found for DOPAC and HVA (Fig. 9). These data demonstrate that rottlerin has the potential to rescue MPTP-induced dopaminergic neurotoxicity in animal PD models.

**Discussion**

The present study demonstrates that the PKCβ inhibitor rottlerin protects against MPTP-induced motor deficits, striatal dopamine depletion, and nigral dopaminergic neuronal loss. The neuroprotective effect of rottlerin was also evident against dopaminergic neurodegeneration, because the inhibitor rescued TH⁺ neurons from MPP⁺-induced neurotoxicity in primary mesencephalic cultures. To our knowledge, this is
the first demonstration of a neuroprotective strategy using a pharmacological inhibitor of PKC\(\beta\) in an animal model of Parkinson’s disease.

The PKC family of kinases consists of 13 isoforms classified into three distinct subfamilies based on their activation profiles. Conventional isoforms of PKCs, PKC\(\alpha\), -\(\beta\), -\(\gamma\), and -\(\delta\), require both intracellular calcium and diacylglycerol for their activation, whereas the novel PKC isoforms, PKC\(\zeta\), -\(\epsilon\), -\(\eta\), -\(\theta\), and -\(\mu\), require only diacylglycerol. The atypical PKCs, PKC\(\epsilon\) and -\(\lambda\) \((\kappa)\), require neither calcium nor phospholipids for activation. The expression patterns and biological functions of these isoforms in the CNS are only beginning to be recognized. For example, PKC\(\gamma\) is expressed predominantly in the brain, and it has been implicated in neural plasticity, including long-term potentiation (Saito and Shirai, 2002) and ischemic injury (Aronowski and Labiche, 2003). The levels of another novel PKC isoform, PKC\(\varepsilon\), are greatly reduced in Alzheimer’s brains, and they are linked to increased production of amyloid \(\beta\) protein (Akita, 2002). In the CNS, PKC\(\epsilon\) is localized mainly in the presynaptic region and induces neurite outgrowth during differentiation and mediates apoptosis during embryonic development, among other functions (Akita, 2002).

Recent studies from our laboratory and those of others have demonstrated that PKC\(\delta\), a key member of novel PKCs, plays a proapoptotic role in various cell types (Kanthasamy et al., 2003). We also showed that PKC\(\delta\) is an oxidative stress kinase in the CNS (Kanthasamy et al., 2003; Kaul et al., 2005). Oxidative stress and apoptotic cell death have been implicated in several neurodegenerative disorders, including Parkinson’s disease (Maguire-Zeiss et al., 2005; West et al., 2005; Smith et al., 2006). We showed that oxidative stress, in dopaminergic neuronal models, persistently activates PKC\(\delta\) by proteolysis via caspase-3 cleavage of the native kinase (72–74 kDa), which yields a 41-kDa catalytically active fragment and a 38-kDa regulatory fragment (Anantharam et al., 2002; Kitazawa et al., 2003). We also demonstrated that the proteolytic activation of PKC\(\delta\) contributes to apoptotic cell death in cell culture models of PD (Kaul et al., 2003; Yang et al., 2004). In addition to the proapoptotic role, PKC\(\delta\) amplifies apoptotic signaling via positive feedback activation of the
inactive mutants, PKC Δamplifier of apoptosis. Overexpression of the dominant-negative and PKC inhibitors (Gschwendt et al., 1994; Samokhin et al., 1999; Davies et al., 2000; Soltoff, 2001). Studies have also shown rottlerin to inhibit other kinases such as calmodulin kinase III, p38 regulated/activated protein kinase, and mitogen-activated protein kinase-activated protein kinase 2 (Gschwendt et al., 1994; Samokhin et al., 1999; Davies et al., 2000; Soltoff, 2001). Toxicity data for rottlerin indicate that the compound has a low toxicity profile (lowest lethal dose = 750 mg/kg, rat oral); 120 mg/kg (oral 6-day rat study) is the lowest toxic dose (Varma et al., 1959). The relative safety, combined with its efficacy, makes rottlerin quite attractive as a potential therapeutic agent.

Previous studies have shown that rottlerin treatment rescues non-neuronal cells from apoptotic death induced by various stimuli (Kanthasamy et al., 2003); in addition, rottlerin inhibits PKCδ enzyme activity in vitro (Anantharam et al., 2002). Recently, we showed that MPP⁺-induced increases in PKCδ kinase activity were effectively blocked by rottlerin treatment in an immortalized dopaminergic mesencephalic neuronal cell line (Kaul et al., 2003). In the present study, we provide evidence that rottlerin treatment can also rescue TH⁺ neurons from MPP⁺-induced neurotoxicity in primary mesencephalic cultures. Extension of these studies in animal models revealed rottlerin can effectively attenuate the MPTP-induced increase in PKCδ kinase activity in mouse SNc. Furthermore, rottlerin administration attenuated neurochemical depletion and locomotor activity of MPTP-treated animals, demonstrating protection against both behavioral and neurochemical deficits. Rottlerin treatment also protected against MPTP-induced loss of TH neurons in the SNc, revealing the neuroprotective effect in a well-characterized preclinical model of PD. Taken together, the observed protective effect of the PKCδ inhibitor rottlerin in an MPTP model of PD strongly supports our previous mechanistic studies demonstrating proapoptotic function of PKCδ in dopaminergic neuronal cell death (Kaul et al., 2003, 2005; Yang et al., 2004).

A number of researchers have attempted to develop neuroprotective agents targeting cell death signaling molecules. Inhibitors targeted against mixed lineage kinase and poly-(ADP-ribose) polymerase have been shown to protect nigral dopaminergic neurons in animal models (Iwashita et al., 2004), and some of these agents are currently being evaluated in human clinical trials (Parashar et al., 2005). Previous studies have shown that PKCδ can indirectly regulate poly-(ADP-ribose) polymerase and mixed lineage kinase (Yoshida et al., 2002; Kitazawa et al., 2004), suggesting that PKCδ
may be an attractive upstream neuroprotective therapeutic target. Saporito et al. (1999) showed systemic injection of the c-Jun NH\textsubscript{2}-terminal kinase inhibitor CEP-1347/KT-7515 (Maroney et al., 1998) protected against MPTP toxicity in the C57 black mouse model. However, CEP-1347 was effective only in the pretreatment regimen and not in the postexposure period to MPTP. The outcome of recent clinical trials with the CEP-1347 compound was not encouraging due to the lack of clinical improvement in PD patients, which may be related to the failure of the compound in the preclinical model during post-treatment to prevent the progression of the disease (Burke, 2007). In the present study, rottlerin post-treatment protects against degenerative processes even 3 days after MPTP exposure, demonstrating its neuroprotective properties. Normally, PD patients lose around 70% of dopaminergic innervation before they develop signs of PD; therefore, the effective rottlerin post-treatment regimen shows real promise for therapeutic development. Advantageously, rottlerin is orally active.

Maruyama et al. (2002) developed a class of compounds with multiple putative properties, including iron chelation, monoamine oxidase inhibition, and antioxidant effects (Maruyama et al., 2002), but the preclinical and clinical evaluations of these compounds have not yet been recently evaluated. Recent phase II clinical trials with 12 compounds have demonstrated creatine and minocycline as effective; these compounds have been recommended to proceed to phase III trials to determine whether they alter the long-term progression of PD (National Institute of Neurological Disorders and Stroke, 2006). Minocycline, a tetracycline derivative with anti-inflammatory properties, prevented dopaminergic neurodegeneration in MPTP models of PD (Du et al., 2001). Minocycline also inhibits microglial-associated inflammation and apoptosis (Ravina et al., 2003; Kelly et al., 2004). Oral administration of the nutritional supplement creatine attenuated MPTP toxicity, which is similar to our findings with rottlerin (Matthews et al., 1999). Creatine exhibits neuroprotective properties by inhibiting mitochondrial permeabilization as well as by indirectly acting as an antioxidant (Tarnopolsky and Beal, 2001). Very recently, the National Institute of Neurological Disorders and Stroke launched a multicenter, large-scale, 7-year phase III clinical trial, at the cost of approximately $60 million, to evaluate the neuroprotective effect of a purified form of creatine (Couniz, 2007). In addition to creatine, the antioxidant coenzyme-Q is being evaluated in PD clinical trials (Shults and Haas, 2005). Release of cytochrome c resulting from increased mitochondrial permeability as well as oxidative stress has been shown to initiate PKC\textsuperscript{\textalpha} activation (Anantharam et al., 2002), and antioxidant treatment attenuates proapoptotic action of the kinase (Kaul et al., 2003), indicating that PKC\textsuperscript{\textalpha} is closely associated with two leading PD pathological mechanisms, i.e., oxidative insult and mitochondrial dysfunction.

In conclusion, we demonstrate that inhibition of a PKC\textsuperscript{\textalpha} isoform with rottlerin can offer protection against behavioral deficits, neurochemical depletion, and nigral dopaminergic neuronal damage in animal models of PD. Our results provide evidence that PKC\textsuperscript{\textalpha} may serve as a novel therapeutic target for development of neuroprotective agents, and they suggest that development of selective and systemically active small molecule PKC\textsuperscript{\textalpha} isoform inhibitors may translate to an effective neuroprotective agent for treatment of PD.


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