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Protein Kinase C δ Upregulation in Microglia Drives Neuroinflammatory Responses and Dopaminergic Neurodegeneration in Experimental Models of Parkinson's Disease

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Abstract

Chronic microglial activation has been linked to the progressive degeneration of the nigrostriatal dopaminergic neurons evidenced in Parkinson's disease (PD) pathogenesis. The exact etiology of PD remains poorly understood. Although both oxidative stress and neuroinflammation are identified as co-contributors in PD pathogenesis, signaling mechanisms underlying neurodegenerative processes have yet to be defined. Indeed, we recently identified that protein kinase C δ (PKC δ) activation is critical for induction of dopaminergic neuronal loss in response to neurotoxic stressors. However, it remains to be defined whether PKC δ activation contributes to immune signaling events driving microglial neurotoxicity. In the present study, we systematically investigated whether PKC δ contributes to the heightened microglial activation response following exposure to major proinflammatory stressors, including α -synuclein, tumor necrosis factor α (TNF α), and lipopolysaccharide (LPS). We report that exposure to the aforementioned inflammatory stressors dramatically upregulated PKC δ with a concomitant increase in its kinase activity and nuclear translocation in both BV-2 microglial cells and primary microglia. Importantly, we also observed a marked upregulation of PKC δ in the microglia of the ventral midbrain region of PD patients when compared to age-matched controls, suggesting a role for microglial PKC δ in neurodegenerative processes. Further, shRNA-mediated knockdown and genetic ablation of PKC δ in primary microglia blunted the microglial proinflammatory response elicited by the inflammogens, including ROS generation, nitric oxide production, and proinflammatory cytokine and chemokine release. Importantly, we found that PKC δ activated NF κ B, a key mediator of inflammatory signaling events, after challenge with inflammatory stressors, and that transactivation of NF κ B led to translocation of the p65 subunit to the nucleus,

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Conflict of Interest A.G.K. and V.A. are shareholders of PK Biosciences Corporation (Ames, IA), which is interested in translating mechanistic studies into therapies targeting kinases including PKC δ . The other authors have no conflicts of interest.

I κ B α degradation and phosphorylation of p65 at Ser536. Furthermore, both genetic ablation and siRNA-mediated knockdown of PKC δ attenuated NF κ B activation, suggesting that PKC δ regulates NF κ B activation subsequent to microglial exposure to inflammatory stimuli. To further investigate the pivotal role of PKC δ in microglial activation *in vivo*, we utilized pre-clinical models of PD. We found that PKC δ deficiency attenuated the proinflammatory response in the mouse substantia nigra, reduced locomotor deficits and recovered mice from sickness behavior in an LPS-induced neuroinflammation model of PD. Likewise, we found that PKC δ knockout mice treated with MPTP displayed a dampened microglial inflammatory response. Moreover, PKC δ knockout mice exhibited reduced susceptibility to the neurotoxin-induced dopaminergic neurodegeneration and associated motor impairments. Taken together, our studies propose a pivotal role for PKC δ in PD pathology, whereby sustained PKC δ activation drives sustained microglial inflammatory responses and concomitant dopaminergic neurotoxicity consequently leading to neurobehavioral deficits. We conclude that inhibiting PKC δ activation may represent a novel therapeutic strategy in PD treatment.

Keywords

Microglial activation; PKC δ ; NF κ B; neuroinflammation; α -synuclein; PKC delta; oxidative stress; Parkinson's disease; dopaminergic degeneration

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting around 2% of the population over the age of 60 (Moore et al., 2005; Schapira, 2009). The pathological hallmarks of PD include a profound loss of dopaminergic neurons in the substantia nigra, resulting in the depletion of striatal dopamine that is clinically manifested as a triad of cardinal motor impairments that include rigidity, bradykinesia and tremors (Obeso et al., 2010). Extranigral neuronal loss and non-motor symptoms have also been recognized in recent years (Beitz, 2014; Bohnen et al., 2014). Interestingly, elevated glial activation is evident during the early stages of PD pathogenesis that preceded dopaminergic degeneration and remains apparent in postmortem PD patients (Block et al., 2007; Glass et al., 2010; Hirsch and Hunot, 2009; Tansey and Goldberg, 2010). Compelling evidence from human, animal and epidemiological studies now supports a pathogenic role for chronic neuroinflammation during dopaminergic degeneration (Appel et al., 2010; Benner et al., 2004; Brochard et al., 2009; Ghosh et al., 2007b; Tansey and Goldberg, 2010; Wu et al., 2002). The substantia nigra has the highest microglial density as compared to other brain regions (Yang et al., 2013). It has been suggested that an increased number of microglia as well as higher levels of TNF α receptor 1 in the substantia nigra make it more vulnerable to systemic immunological insults (Yang et al., 2013). However, little is known about the signaling pathways regulating sustained microglial activation or the process by which microglial cells evade regulatory control to become persistently activated, thereby contributing to progressive dopaminergic degeneration. Microglial NADPH oxidase is involved in generating free radicals in the substantia nigra and can exacerbate dopaminergic degeneration by augmenting local oxidative stress (Wu et al., 2002; Wu et al., 2003). Similarly, NF κ B activation in microglia was shown to be a critical regulator of the

microglial neurotoxic response by regulating proinflammatory cytokine levels and iNOS activity in the substantia nigra (Ghosh et al., 2007b). However, the intracellular signaling pathways underlying sustained microglial activation and its link to dopaminergic degeneration in PD pathogenesis remain enigmatic.

Protein kinase C δ (PKC δ), a member of the novel PKC isoform family, is involved in numerous signal transduction pathways related to proliferation, cell cycle progression, differentiation, and apoptosis. We previously reported that the kinase is highly expressed by dopaminergic neurons in the SNpc, primary dopaminergic cultures and dopaminergic cell lines (Zhang et al., 2007a). Studies from our laboratory have shown that PKC δ is proteolytically cleaved by caspase-3 during neurotoxic stress in dopaminergic cells undergoing apoptotic neuronal cell death (Anantharam et al., 2002; Hanrott et al., 2008; Kaul et al., 2003; Zhang et al., 2007a). Recently, we reported that stimulation of TNF α receptors in dopaminergic neurons can promote apoptosis via proteolytic activation of PKC δ (Gordon et al., 2012). Although PKC δ proteolytic activation has been demonstrated in dopaminergic neurons, its role in glial activation during inflammatory insults is not well characterized. PKC δ has also been shown to regulate inflammatory responses and immune signaling in peripheral immune cells including neutrophils, B cells and macrophages (Bey et al., 2004; Carpenter and Alexander, 2008; Kilpatrick et al., 2010; Mecklenbrauker et al., 2004; Tepperman et al., 2000).

The precise role of PKC δ in microglial activation and subsequent microglia-driven dopaminergic neurotoxicity remains poorly understood. Therefore, in the current study we determined the magnitude of PKC δ activation states in primary microglia and BV-2 microglial cells following exposure to diverse inflammogens (TNF α , aggregated α -synuclein and LPS). Additionally, we examined whether pharmacological inhibition, gene silencing and genetic ablation of PKC δ attenuated microglia-activated inflammatory response. Furthermore, we extended our *in vitro* findings to LPS and MPTP mouse models of PD and to the ventral mesencephalon of human PD brains. We initially suspected that PKC δ could be proteolytically activated in microglial cells, similar to dopaminergic neurons. However, to our surprise, we found that PKC δ expression and kinase activity were elevated in activated microglia in the absence of PKC δ cleavage. Herein, we demonstrate that PKC δ directly regulates the microglial inflammatory response and nigrostriatal dopaminergic degeneration in both *in vitro* and *in vivo* models of PD. Our findings suggest a pivotal role for PKC δ in neuroinflammation and dopaminergic neurotoxicity.

Materials and Methods

Chemicals and reagents

DMEM/F-12, RPMI, fetal bovine serum (FBS), L-glutamine, IR-dye tagged secondary antibodies, Hoechst nuclear stain, penicillin, streptomycin and other cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). We purchased recombinant TNF α from Peprotech (Rocky Hill, NJ), LPS (*E. coli* 0111:B4) from Sigma and recombinant human α -synuclein from rPeptide (Bogart, GA). Aggregation of human α -synuclein was performed according to previously published procedures (Zhang et al., 2005) by incubating the purified recombinant protein at RT with agitation for 7 days. The formation of human α -

synuclein aggregates was verified by transmission electron microscopy (Supplementary Fig. 1). Human α -synuclein was used instead of murine α -synuclein because it is structurally and biochemically well characterized. The advantages of using human α -synuclein also include the availability of human-specific α -synuclein antibodies, which enables the identification of exogenously applied α -synuclein (Volpicelli-Daley et al., 2014). Moreover, the protocols for α -synuclein aggregation have been established and reproduced by multiple groups using human α -synuclein (Fellner et al., 2013; Kim et al., 2013; Lee et al., 2010; Zhang et al., 2005). Rabbit anti-PKC δ and anti-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-TH antibody was purchased from Chemicon (Temecula, CA), p65, p-p65 and p-I κ B α antibodies were purchased from Cell Signaling (Danvers, MA) and rabbit anti-Iba1 antibody was from Wako Chemicals (Richmond, VA). 32 P-ATP was purchased from Perkin Elmer (Boston, MA) and the histone substrate from Sigma. The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

Animal studies

PKC δ knockout (KO) mice were generated and maintained as described previously (Zhang et al., 2007a). Mice were genotyped according to previously published protocols (Miyamoto et al., 2002). Wild-type (WT) C57BL/6 and PKC δ KO mice were housed under standard conditions of constant temperature ($22 \pm 1^\circ\text{C}$), humidity (relative, 30%), and a 12-h light cycle with *ad libitum* access to food and water. Six- to eight-week-old mice were used for all studies. The well-characterized acute MPTP mouse model of PD (Hu et al., 2008; Kim et al., 2007; Przedborski et al., 2004; Wu et al., 2003) was used for neuroinflammation and neuroprotection studies because the kinetics of microglial neuroinflammation is well characterized in this model. The mice from the MPTP treatment group received 4 i.p. injections of MPTP-HCl (18 mg/kg free base) at 2 h intervals and control mice received equivalent saline injections. The mice were sacrificed 1 to 7 days after the last injection. The nigral neuroinflammatory response was also studied using the systemic LPS injection model, used for inducing chronic neuroinflammation and delayed dopaminergic degeneration in mice (Lee et al., 2008; Qin et al., 2007). A single injection of LPS (5 mg/kg, i.p.) was delivered to PKC δ WT and KO mice. Control groups received equivalent injections of saline. Mice were sacrificed 24 to 48 h later. For animal euthanasia, we utilized a standard anesthetic regimen consisting of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). This anesthetic combination has been widely used in several neurosurgical procedures and cited numerous times in the neurodegeneration literature by several groups in addition to our previous publications (Jin et al., 2011; Panicker et al., 2015; Saijo et al., 2009). This anesthetic mixture does not significantly affect the phosphorylation status of PKC δ or other kinases that we have studied to date. All animal procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

Primary microglial cultures and treatments

Primary microglial cultures were prepared from C57BL/6 postnatal day 1 (P1) mouse pups. Mouse brains were harvested and their meninges removed before being placed in Dulbecco's modified Eagle's medium/F-12 nutrient media (DMEM-F12, GIBCO Cat# 11320) supplemented with 10% heat-inactivated FBS, 50 U/mL penicillin, 50 $\mu\text{g/mL}$ streptomycin,

2 mM L-glutamine, 100 μ M non-essential amino acids, and 2 mM sodium pyruvate (Invitrogen). Brain tissues were then incubated in 0.25% trypsin for 30 min with gentle agitation. The trypsin reaction was stopped by adding an equal volume of DMEM/F12 complete medium and the brain tissues were washed 3 times. Tissues were then triturated gently to prepare a single-cell suspension and then passed through a 70- μ m nylon mesh cell strainer to remove tissue debris and aggregates. Cells were re-suspended in DMEM/F12 complete medium and seeded into T-75 flasks, which were placed in a humidified CO₂ incubator at 37°C. The medium was changed after 4 to 5 days and the mixed glial cells were grown to confluence. Microglial cells were separated from confluent mixed glial cultures by differential adherence and magnetic separation to >97% purity as described in our recent publication (Gordon et al., 2011). Microglia were allowed to recover for at least 48 h after plating. Primary microglia were treated in DMEM/F12 complete medium containing 2% FBS.

Lentiviral shRNA- and siRNA-mediated knockdown of PKC δ in primary microglia

Lentiviral transduction and knockdown of PKC δ in primary microglia was performed according to previously published reports (Jin et al., 2014; Takahashi et al., 2005) with some modifications. Pre-validated lentiviral particles expressing PKC δ -specific shRNA were purchased from Santa Cruz Biotechnology (Cat# sc-36246-V) along with non-specific control shRNA lentiviral particles (Cat# sc-108080). Titers of the viral particles were around 5×10^6 infectious units (IFU)/ml. Purified microglial cells were seeded at 3×10^5 cells/ml into 24-well plates and allowed to attach overnight. The next day, lentiviral particles (MOI = 2) and 5 μ g/ml of polybrene (Millipore) were added to each well and the plates were centrifuged for 60 min at 30°C. The plates were further incubated for 3 h at 37°C before replacing the supernatant containing the viral particles with complete DMEM/F12 medium containing 10% FBS. The efficiency of transduction was around 90% as determined by GFP expression 48 h later. Lentiviral shRNA knockdown of the PKC δ protein in primary microglia was confirmed by Western blotting using a rabbit polyclonal PKC δ antibody. For siRNA-mediated PKC δ knockdown studies, silencer select PKC δ siRNA (Cat# 4390771) and scrambled siRNA were purchased from Invitrogen. PKC δ knockdown was performed using the Mouse Macrophage Nucleofector kit from Lonza (Cat# VPA-1009). Briefly, $2-3 \times 10^6$ primary microglia were suspended in 100 μ l of nucleofection reagent and mixed thoroughly with 1.5 μ g PKC δ or scrambled siRNA. The mixture was transferred to an electroporation cuvette and electroporated using the A-23 program in the Nucleofector I device. The transfected microglia were resuspended in 500 μ l warmed media and incubated in 6-well plates for 48 h. The PKC δ knockdown efficiency was further confirmed by Western blot (Supplementary Fig. 2).

Immunohistochemistry and immunofluorescence studies

Autopsy brain tissues from three male PD patients and four control subjects were obtained from Dr. Asgar Zaheer's lab in the University of Iowa. PD patients and control subjects did not differ significantly for their mean age at death (PD, 74 ± 5 years; control, 78 ± 8 years). Additional age-matched postmortem substantia nigra sections from two controls and two PD subjects were obtained from the Queensland Brain Bank (Brisbane, Australia). Antigen retrieval for human sections was performed by heating sections at 98°C for 45 min in 0.05%

citraconic anhydride solution (pH 7.4). Mouse substantia nigra sections were processed for immunohistochemistry followed by antigen retrieval by heating the free-floating sections in citrate buffer (10 mM sodium citrate, pH 8.5) for 30 minutes at 90°C. Sections were then washed several times in PBS and blocked with PBS containing 2% BSA, 0.2% Triton X-100 and 0.05% Tween-20 for 1 h at RT and incubated with the respective primary antibodies overnight at 4°C. Primary antibodies used were PKC δ (goat polyclonal, 1:500), TH (mouse monoclonal, 1:2000) and Iba1 (rabbit polyclonal, 1:500). The sections were then incubated with Alexa dye-conjugated secondary antibodies for 1 h at RT. Sections were dehydrated by rising concentration gradient of ethanol followed by xylenes, and coverslipped with Permount or DPX. For human postmortem studies, sections were treated using the Autofluorescence Eliminator Reagent (Merck Millipore, Catalog number 2160) according to the manufacture's instructions to eliminate lipofuscin-derived autofluorescence. Briefly, sections were washed in PBS after the secondary antibody step followed by immersion in 70% ethanol for 5 minutes. Sections were then treated with Autofluorescence Eliminator Reagent for 5 minutes followed by three changes of ethanol for 1 minute each. Sections were then mounted using Prolong Gold antifade reagent (Molecular Probes). Samples were visualized using a Leica DMIRE2 confocal microscope or Nikon inverted fluorescence microscope (TE-2000U) and images were captured using a SPOT digital camera (Diagnostic Instruments Inc). PKC δ and p65 immunofluorescence in primary microglia was performed according to previously published protocols (Ay et al., 2015; Jin et al., 2015), with some modifications. Briefly, microglial cells were grown on poly-D-lysine-coated coverslips and treated 48 h later. At the end of treatments, cells were fixed with 4% PFA, washed in PBS and incubated in blocking buffer (PBS containing 2% BSA, 0.5% Triton X-100 and 0.05% Tween-20) for 1 h at RT. The coverslips were then incubated overnight at 4°C with primary antibodies (rabbit PKC δ , 1:500 or rabbit p65, 1:500) diluted in PBS containing 2% BSA. Samples were then washed several times in PBS and incubated with Alexa dye-conjugated secondary antibodies. The nucleus was labeled with Hoechst stain and coverslips were mounted with Fluoromount medium (Sigma Aldrich) on glass slides for visualization using a confocal microscope.

qRT-PCR for gene expression analysis

RNA isolation from primary microglial cells and brain tissue samples was performed using the Absolutely RNA Miniprep kit, and 1 μ g of total RNA was used for reverse transcription with the Affinity Script qPCR cDNA synthesis system (Agilent Technologies) according to the manufacturer's instructions. Quantitative SYBR Green real-time PCR assays for PKC δ and proinflammatory cytokine gene expression analysis were performed using the RT² SYBR Green Master Mix with pre-validated qPCR primers (SABioscience qPCR assay system). Catalog numbers of the primers were PKC δ - PPM03810E, IL-1 β - PPM03109E, IL-12 - PPM03020E, TNF α - PPM03113F and iNOS - PPM02928B. The mouse 18S rRNA gene (Cat# PPM57735E) was used as the housekeeping gene for normalization. For each primer, the amount of template which provided the maximum efficiency without inhibition of the PCR reaction was determined during initial optimization experiments. For all experiments, dissociation curves were run according to the manufacturer's instructions to ensure a single peak was obtained at the expected melting temperature without non-specific amplicons. Fold change in gene expression was determined by the C_t method using the

threshold cycle (C_t) value for the housekeeping gene and for the respective gene of interest for each sample.

Western blotting

Brain tissue and microglial cell lysates were prepared using a modified RIPA buffer and normalized for equal amounts of protein using the Bradford protein assay kit. Equal amounts of protein (30–50 μ g) were loaded for each sample and separated on either 12% or 15% SDS-PAGE gels based on the molecular weight of the target protein. After separation, proteins were transferred to a nitrocellulose membrane and the nonspecific binding sites were blocked for 1 h using a blocking buffer specifically formulated for fluorescent Western blotting (Rockland Immunochemicals). Membranes were then probed overnight at 4°C with the respective primary antibodies rabbit polyclonal PKC δ (1:1000), mouse monoclonal TH (1:2000), rabbit polyclonal Iba1 (1:200), and mouse monoclonal gp91phox (1:2000). After incubation, the membranes were washed 3 times with PBS containing 0.05% Tween-20 and IR-dye tagged secondary antibodies (1:4000) were added and the membranes visualized on the Odyssey infrared imaging system; β -actin and tubulin (1:5000) were used as loading controls.

PKC δ immunoprecipitation-kinase assays

PKC δ IP kinase activity assays were performed as described previously (Harischandra et al., 2014; Kaul et al., 2005; Zhang et al., 2007a) with some modifications for microglial cells. Briefly, primary microglia or BV-2 microglial cells were collected after treatments, washed in ice-cold PBS and resuspended in a mild RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (Pierce Biotechnology). The cells were placed on ice for 20 min to allow for complete lysis and then centrifuged at 13,000 rpm for 45 min. The supernatant was collected and protein concentration was determined using the Bradford assay (Bradford, 1976). The samples were normalized to a uniform total protein concentration of 2 μ g/ μ l, and then 500 μ g of total protein in a 250 μ l reaction volume was immunoprecipitated overnight at 4°C using 5 μ g of PKC δ antibody. The next day, protein-A agarose beads (Sigma Aldrich) were added and the samples were incubated for 1 h at RT. The protein A-bound antibody complexes were collected and washed 3 times in 2X kinase assay buffer (40 mM Tris, pH 7.4, 20 mM MgCl₂, 20 μ M ATP, and 2.5 mM CaCl₂) and then resuspended in the same buffer. The kinase reaction in the immunoprecipitated samples was started by adding 40 μ l of the reaction buffer containing 0.4 mg of histone H1, 50 μ g/ml phosphatidylserine, 4 μ M dioleoylglycerol, and 10 μ Ci of [γ -³²P] ATP at 3000 Ci/mM. The samples were then incubated for 10 min at 30°C and the kinase reaction was stopped by adding 2X SDS loading buffer and boiling for 5 min. Proteins were separated on a 15% SDS-PAGE gel and the phosphorylated histone bands were imaged using a Fujifilm FLA 5000 imager. Image analysis and band quantification were performed using Fujifilm Multigauge software.

Microglial nitric oxide detection

Nitric oxide production by primary microglia was measured indirectly by quantification of nitrite in the supernatant using the Griess reagent (Sigma Aldrich). Magnetically separated microglia were plated in poly-D-lysine-coated 96-well plates at 10⁵ cells per well and

treated with 100 ng/mL LPS. After 24 h post-LPS, 100 μ L of supernatant from each well was mixed with an equal volume of Griess reagent in a separate 96-well plate. The samples were incubated on a plate shaker at RT for 15 min until a stable color was obtained. The absorbance at 540 nm was measured using a Synergy 2 multi-mode microplate reader (BioTek Instruments) and the nitrite concentration was determined from a sodium nitrite standard curve.

Multiplex cytokine and chemokine Luminex immunoassays

Primary microglia were obtained from PKC δ WT and KO mice on the same day and seeded in poly-D-lysine-coated 96-well plates at 0.5×10^5 cells per well. The cells were treated for 24 h with either 100 ng/mL LPS, ng/ml TNF α or 300 nM aggregated α -synuclein. After treatment, 50 μ L of supernatant from each well was collected and frozen at -80°C . The levels of cytokines and chemokines in supernatants were determined using the Luminex bead-based immunoassay platform (Vignali, 2000) using pre-validated multiplex kits (Milliplex mouse cytokine and chemokine panels – Millipore Corp) according to the manufacturer's instructions.

Intracellular reactive oxygen species (iROS) detection

We determined iROS using the fluorescent probe 2',7'-dichlorodi-hydrofluorescein diacetate (DCFH-DA) (Calbiochem), according to previously published reports (Harischandra et al., 2015; Qian et al., 2007; Zhang et al., 2005). Magnetically separated microglia from PKC δ WT and KO mice were plated at 0.5×10^5 cells per well in 96-well plates and allowed to attach overnight. Before treatment, cells were washed in HBSS and incubated with 40 μ M DCFH-DA in HBSS containing 2% FBS for 30 min. Cells were then treated for 6 h with either 100 ng/mL LPS, 30 ng/mL recombinant mouse TNF α or 300 nM aggregated α -synuclein. The fluorescence intensity was determined using a Synergy-2 multi-mode microplate reader at an excitation of 485/20 nm and an emission of 530/25 nm. The fluorescence value from the control cultures was subtracted as background and the increase in fluorescence with treatments was expressed as increased iROS as previously described (Qian et al., 2007; Zhang et al., 2005).

Behavioral studies and locomotor activity measurements

Five days after MPTP treatment, WT and KO mice were tested for behavioral parameters and locomotor activity. An automated instrument (AccuScan, model RXYZCM-16, Columbus, OH, USA) was used to monitor the locomotor activity of mice. Prior to behavioral studies, mice were placed inside the infrared monitor for 5 min daily for 3 consecutive days to adapt them to the apparatus. The various locomotor activity parameters were determined simultaneously over a 10 min testing session and were presented as horizontal movements, vertical movements, total distance travelled (cm), total movement time, and total rest time. Data were collected and analyzed by a VersaMax Analyzer (AccuScan, model CDA-8, Columbus, OH). For Rotarod performance analysis, a 20-rpm speed setting was used to monitor the foot movement of mice as described in our previous publication (Ghosh et al., 2013). A total of 6 trials per mouse was performed and mice were given a 7–10 min rest interval between trials.

Tyrosine hydroxylase-diaminobenzidine immunostaining and stereological counting of TH-positive neurons

TH-diaminobenzidine (DAB) immunostaining was performed on striatal and substantia nigral sections as described previously (Ghosh et al., 2010). Briefly, PKC δ WT and KO mice were injected with MPTP or saline and sacrificed 7 days after treatment. The mice were perfused with 4% PFA and brains were post-fixed with 4% PFA for 48 h before storage in 30% sucrose. Frozen blocks were prepared from the fixed brains and were cut using a cryostat into 30- μ m coronal sections. Sections were probed with an anti-TH antibody (Calbiochem; rabbit anti-mouse, 1:1600) overnight at 4°C and then incubated with biotinylated anti-rabbit secondary antibody. The sections were then treated with Avidin peroxidase (Vectastain ABC Elite kit). The DAB reagent provided with the kit was used for producing the brown colored stain. The total number of TH-positive neurons in the SNpc was counted by unbiased stereology using Stereo Investigator software (MicroBrightField, Inc., Williston, VT) and the optical fractionator method as described in our previous publications (Ghosh et al., 2010; Ghosh et al., 2007a).

HPLC analysis of striatal dopamine and its metabolite levels

The amount of striatal dopamine and its metabolites (DOPAC and HVA) was quantified by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Ghosh et al., 2010; Zhang et al., 2007b). Briefly, 7 days after the last MPTP injection, PKC δ WT and KO mice were sacrificed and their striata were collected, weighed and stored at -80°C. Neurotransmitters from striatal tissues were extracted in a 0.1 M perchloric acid solution containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅ and isoproterenol (used as an internal standard). Extracts were filtered in 0.22- μ m spin tubes, and 20 μ l of the sample was loaded for analysis. Dopamine, DOPAC and HVA were separated isocratically in a reversed-phase column using a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA, USA) with a thermostatted autosampler (model 542; ESA Inc.) was used for all experiments. The electrochemical detection system consists of a Coulochem model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). Standard catecholamine stock solutions of 1 mg/ml were prepared in perchloric acid solution and further diluted to a final working concentration of 50 pg/ μ l prior to injection. All data acquisition and analysis were performed using EZStart HPLC Software (ESA Inc.).

Data analysis

Data analysis was performed using Prism 4.0 (GraphPad Software, San Diego, CA). Data were first analyzed using one-way ANOVA and then Bonferroni's post-test was performed to compare all treatment groups. Differences of $p < 0.05$ were considered statistically significant. The Student's t-test was used when comparing differences between two groups.

Results

PKC δ upregulation in response to multiple inflammatory stressors during microglial activation

Previous studies from our laboratory demonstrated that proteolytic activation of PKC δ occurs during dopaminergic degeneration and is mediated by caspase-3 (Anantharam et al., 2002; Kanthasamy et al., 2003; Kaul et al., 2003; Sun et al., 2008; Zhang et al., 2007a). To establish the role of microglial PKC δ in microglial activation, first we determined PKC δ protein expression levels and proteolytic activation following treatment with well-known inflammogens including LPS and TNF α . We also examined if aggregated α -synuclein, a PD-specific inflammogen known to activate microglia during PD pathology (Boza-Serrano et al., 2014; Lema Tome et al., 2013; Valera and Masliah, 2013), affects PKC δ protein expression levels and proteolytic activation. Using an antibody directed against the C-terminus of the protein in primary microglia and BV-2 microglial cells, surprisingly, we did not detect a significant increase in the 41-kDa proteolytically cleaved PKC δ product during microglial activation with any of the inflammogen treatments. Instead, protein levels of 74-kDa native full-length PKC δ was highly upregulated during sustained microglial activation. The full-length PKC δ protein was significantly upregulated in mouse primary microglia at both 12 and 24 h following treatment with 100 ng/ml LPS, 30 ng/ml TNF α and 300 nM aggregated α -synuclein (Fig. 1A). A similar response was also evidenced in LPS- and TNF α -treated BV-2 cells, a commonly used microglial cell line (Fig. 1B). To determine whether increased PKC δ protein expression was due to increased transcription, we used qRT-PCR to examine PKC δ gene expression 24 h after treating primary microglia with LPS, TNF α or aggregated α -synuclein. We observed significant increases (3–6 fold) in PKC δ mRNA levels in activated microglia (Fig. 1C). TNF α caused the highest increase in PKC δ mRNA levels followed by LPS and aggregated α -synuclein. LPS also induced a time-dependent increase of PKC δ mRNA from 4–24 h in BV-2 cells (Fig. 1D). Together, these results demonstrate for the first time that PKC δ mRNA and protein levels are upregulated during microglial activation following stimulation with inflammogens including aggregated α -synuclein.

We further determined if the increase in PKC δ protein levels was associated with a concomitant increase in PKC δ kinase activity. To this end, we performed immunoprecipitation kinase assays with a histone substrate and 32 P-ATP to measure PKC δ kinase activity in primary microglia and BV-2 cells activated with LPS, TNF α and aggregated α -synuclein. We observed only basal PKC δ kinase activity in unstimulated primary microglia, however, stimulation with LPS, TNF α or aggregated α -synuclein significantly increased PKC δ kinase activity (Fig. 1E). LPS-, TNF α - and aggregated α -synuclein-treated primary microglia showed a 7-, 10- and 4-fold increase in PKC δ kinase activity, respectively, when compared to unstimulated control cells. We obtained similar results with BV-2 microglial cells under the same treatment conditions (Fig. 1F).

Next, we validated our findings in animal models of neuroinflammation and neurodegeneration. For these studies, we used the MPTP model, a well-characterized mouse model relevant to neuroinflammation and PD (Czlonkowska et al., 1996; Du et al., 2001;

Przedborski et al., 2004; Wu et al., 2003). A robust microglial activation response has been observed 24 to 48 h after MPTP-induced dopaminergic degeneration in the mouse substantia nigra (Qin et al., 2007; Wu et al., 2003). Therefore, we injected C57BL mice with four doses of 18 mg/kg MPTP at 2-h intervals. Then, 24 h after the final MPTP injection, nigral tissues were collected for qRT-PCR analysis. We observed around a 9-fold increase in PKC δ mRNA in the ventral midbrain of MPTP-treated mice (Fig. 1G). To validate our results obtained using preclinical models of PD, we examined the PKC δ expression in human PD postmortem midbrain sections (Fig. 1H). Nigral regions of midbrain sections from postmortem PD and age-matched controls were stained for microglia (Iba1) (red) and PKC δ (green). Increased PKC δ staining was clearly evident in the midbrain sections of postmortem PD brains, which showed increased colocalization with Iba1-positive microglia (Fig. 1H, lower panel). In contrast, brain sections from age-matched control brains showed very low PKC δ immunofluorescence, suggesting that PKC δ is upregulated in chronically activated microglia present in PD brains. Together, these results demonstrate that PKC δ protein is upregulated upon microglial activation in cell cultures, animal models and human PD brains.

PKC δ translocates to the nucleus during microglial activation

Upon activation, various PKC isoenzymes often translocate from the cytosol either to the plasma membrane or other subcellular organelles (Newton, 1995). Several studies have characterized the dynamic spatial and temporal re-distribution of PKC δ in different cell types and demonstrated that the differential pattern of subcellular localization of PKC δ may be a critical determinant of its cellular function (Gomel et al., 2007; Humphries et al., 2008; Mecklenbrauker et al., 2004). To determine the role of PKC δ in microglial activation, we first performed confocal analysis to visualize the subcellular localization of PKC δ in the LPS-, TNF α - and α -synuclein-treated microglia at 3, 12 and 24 h post-treatment. In unstimulated primary microglia, PKC δ was largely localized to the cytosol in a diffuse distribution pattern (Top panels, Fig 2A–C). However, upon stimulation of primary microglia with LPS, a massive translocation of PKC δ to the nucleus was evident at 12 and 24 h post-stimulation (Fig. 2A). Interestingly, LPS-induced nuclear translocation of PKC δ was accompanied by a dynamic change in microglial morphology characterized by a rounded amoeboid like appearance with short stubby or withdrawn processes. Notably, microglia treated with TNF α (Fig. 2B) and aggregated α -synuclein (Fig. 2C) also showed nuclear localization of PKC δ at 3, 12 and 24 h post treatment, indicating that microglial activation in response to protein aggregates can also promote PKC δ nuclear translocation. To further confirm our immunocytochemical findings, we examined the subcellular localization of PKC δ at 24 h following treatment with LPS, TNF α and aggregated α -synuclein. Western blots of the PKC δ levels in the cytosolic and nuclear fractions displayed a prominent nuclear localization of PKC δ when compared with unstimulated cells (Fig. 2D–E). In a separate study, we investigated whether PKC δ 's subcellular localization is modulated in BV-2 cells in response to LPS or TNF α . Immunofluorescence revealed a nuclear localization pattern of PKC δ in inflammogen-treated cells similar to that expressed in unstimulated microglia (Fig. 2F–G). Likewise, using Western blot analysis, we demonstrated a time-dependent increase in the translocation of PKC δ from the cytoplasm to the nucleus in both LPS- and TNF α -treated BV-2 cells relative to unstimulated cells (Fig.

2H–K). Taken together, these results indicate that exposure to inflammatory stressors induced the nuclear translocation of PKC δ in both primary microglia and BV-2 cells, irrespective of the nature of the stimuli, suggesting that PKC δ is a convergence point for the induction of the downstream inflammatory cell signaling cascade subsequent to microglial activation.

Suppressing PKC δ expression attenuates LPS-induced nitric oxide production and cytokine release

Following observations that PKC δ was highly induced during microglial activation along with a concomitant increase in its kinase activity, we reasoned that the suppression of PKC δ protein levels could be used to determine its functional role in regulating the microglial activation response. We used a lentiviral shRNA system to specifically suppress PKC δ protein levels in primary microglial cells and studied the microglial activation response with LPS stimulation. Successful lentiviral infection of primary microglia was confirmed by the visualization of green fluorescent protein co-expressed with the PKC δ shRNA (Fig. 3A, top panel). We obtained optimal shRNA suppression of PKC δ protein levels around 72 h after lentiviral infection as determined by Western blotting (Fig. 3A, bottom panel). Primary microglia were treated with 100 ng/ml of LPS for 24 h and nitric oxide production was indirectly measured by nitrite levels using the Griess assay. LPS-induced nitric oxide production was significantly attenuated in primary microglia infected with PKC δ shRNA viral particles when compared to non-specific shRNA-infected microglia (Fig. 3B). We also used a multiplex bead-based Luminex assay system to determine the supernatant levels of typical proinflammatory cytokines released from LPS-activated primary microglia. In PKC δ shRNA-infected microglia, the LPS-induced production of IL-6 and TNF α were both reduced by 70–80% when compared to non-specific shRNA-infected microglia (Fig. 3C), indicating a proinflammatory role for PKC δ signaling in microglia. To further verify the proinflammatory function of PKC δ , we treated primary microglia isolated from WT and PKC δ KO mice with LPS (100 ng/ml) for 24 h. Afterwards, nitrite levels in cell culture media were assayed as a measure of nitric oxide production. LPS induced a sharp increase (~7 fold) in nitrite levels in WT microglia, while there was significantly reduced nitric oxide production in PKC δ KO microglia (Fig. 3D). Next, we studied intracellular ROS generation in primary microglia obtained from PKC δ WT and KO mice using DCF-DA fluorescence assay. Both LPS and TNF α increased intracellular ROS generation by approximately 3-fold in WT microglia at 6 h relative to PKC δ KO microglia (Fig. 3E–F). Together, these results further support an important role for PKC δ in regulating important proinflammatory responses in microglia.

PKC δ deficiency attenuates cytokine and chemokine release during α -synuclein-, LPS-, and TNF α -induced microglial activation

The key hallmark of microglial activation is the generation of inflammatory mediators. To test the role of PKC δ in the production of cytokines and chemokines upon microglial activation by diverse inflammatory stimuli, we first analyzed the activation response of WT and PKC δ KO microglia to LPS and aggregated α -synuclein. Both LPS (Fig. 4A–B) and α -synuclein (Fig. 4C–E) induced the production of the key inflammatory mediators TNF α , IL-1 β and IL-6 in a largely time-dependent manner from 8–24 h. Importantly, primary

microglia isolated from PKC δ KO mice produced a dramatic reduction in TNF α , IL-1 β and IL-6 levels when compared to WT microglia during the entire time of α -synuclein treatment period (Fig. 4C–E). PKC δ KO also significantly attenuated LPS-induced TNF α and IL-1 β levels (Fig. 4A–B). In the next set of experiments, primary microglia obtained from WT and PKC δ KO mice were stimulated with LPS, TNF α and aggregated α -synuclein for a 24-h time period. At the end of the 24-h incubation following LPS stimulation, the levels of cytokines and chemokines, including IL-1 α , MIP-1 α , KC (CXCL-1), MIP-2, and IP-10, were reduced in PKC δ KO microglia when compared with LPS-challenged WT microglia, as determined by multiplex Luminex assay (Table 1). Likewise in PKC δ KO primary microglia, the TNF α -induced inflammatory factor production of IL-6, IP-10, KC, MCP-1, MIP-2 and g-CSF were significantly reduced (Table 1). MIP-1 α and MIP-1 β levels were increased in TNF α -treated PKC δ KO cells when compared with TNF α -treated PKC δ WT cells. This could be attributed to the recruitment of distinct downstream effector signaling events in LPS- and TNF-induced microglial activation (TLR-4 versus TNFR). Collectively, these results point to the regulation of microglial cytokine and chemokine levels by PKC δ signaling, supporting the notion that PKC δ functions as an important regulator of microglial activation. These results also raise the possibility that the signaling pathways downstream of PKC δ , such as NF κ B, might at least in part orchestrate the gene and protein expression cascades during microglial activation.

PKC δ regulates inflammogen-induced activation of the NF κ B signaling pathway in activated microglia

NF κ B signaling plays a major role in modulating proinflammatory mediators in a variety of innate immune cells including microglia (Ghosh et al., 2007b; Jongeneel, 1995). Activation of NF κ B may occur through multiple mechanisms. For example, LPS-induced microglial activation has been associated with the phosphorylation and subsequent degradation of cytosolic I κ B α , followed by nuclear translocation of the NF κ B-p65 subunit (Burguillos et al., 2011; Kawai and Akira, 2007). Therefore, in the present study we examined whether PKC δ regulates the inflammogen-induced NF κ B signaling pathway during microglial activation. For this, we first measured LPS- and TNF α -induced I κ B α degradation in primary microglia isolated from both WT and PKC δ KO mice by immunoblotting analysis. While LPS and TNF α treatments induced a substantial amount of I κ B α degradation in WT microglia, this effect was significantly dampened in LPS and TNF α -challenged PKC δ KO microglia (Fig. 5A–B). We confirmed these results in primary WT microglia transfected with PKC δ -specific siRNA to show that PKC δ knockdown attenuated LPS-induced I κ B α degradation (Fig. 5C–D), which is consistent with our findings using PKC δ -deficient microglia. A similar response was evidenced in TNF α -treated microglia in the presence or absence of the PKC δ inhibitor rottlerin (data not shown). Next, using an antibody specific to p65, we demonstrated that while LPS- and TNF α -induced the nuclear translocation of NF κ B-p65 in WT microglia, this response was significantly diminished in PKC δ KO microglia (Fig. 5E–F). Likewise, inhibition of PKC δ activity using PKC δ siRNA knockdown (Fig. 5G) further corroborated our finding, obtained using the PKC δ KO microglia. To further validate the mode of NF κ B activation, we next examined whether p65 phosphorylation at serine 536 in the transactivation domain plays a crucial role in NF κ B activation in primary microglial cells from PKC δ WT and KO mice treated with LPS and

TNF α for 15 min. Examining the levels of phospho-p65 S536 by Western blot analysis (Fig. 6A–B) reveals that LPS and TNF α treatments rapidly phosphorylated p65 Ser536 in WT microglia, while the extent of phospho-p65 Ser536 was markedly attenuated in PKC δ -deficient microglia. To confirm these results, we again knocked down PKC δ expression by transfecting PKC δ WT primary microglia with PKC δ -specific siRNA, this time to examine LPS-induced p65 Ser536 phosphorylation. Consistent with our other findings using PKC δ -deficient microglia, PKC δ knockdown significantly attenuated LPS-induced p65 phosphorylation (Fig. 6C–D). Similarly, these findings were replicated in cells treated with the PKC δ inhibitor rottlerin (Fig. 6E). In summary, these results suggest that the activation of a canonical signaling cascade following microglial activation, by diverse inflammatory stimuli, leads to the PKC δ -dependent activation of NF κ B and presumably the subsequent generation of increased pro-inflammatory mediators in activated microglial cells.

PKC δ knockout mice exhibit reduced systemic and nigral inflammatory responses following LPS stimulation

To validate our results obtained with primary microglia *in vivo* using animal models of PD, we first utilized the systemic LPS model (Qin et al., 2007). In this model, a single LPS injection in mice results in sustained neuroinflammation in the brain and progressive dopaminergic degeneration almost a year later. We injected PKC δ WT and PKC δ KO mice with a single dose of LPS (5 mg/kg, i.p.) and then compared their behavioral and neuroinflammatory responses. To our surprise, the PKC δ KO mice started to recover from behavioral deficits at around 24 h after LPS administration with a dramatic recovery of normal behavioral responses by 48 h, while WT mice displayed endotoxic shock at around 48 h with hunched posture, lethargy and minimal movement. To further quantify the effect of genetic ablation of PKC δ on motor deficits 48 h after LPS injections, we performed extensive behavioral analyses using the VersaMax Analyzer and the Rotarod. Representative motor activity maps (Fig. 7A) tracking the open-field movements of individual mice exemplify the minimal locomotor activity characterizing lethargic LPS-injected WT mice in stark contrast to the more normal locomotor activity of PKC δ KO mice. The data used to generate the maps was also quantified at the group level as discrete counts of horizontal and vertical activity, which reinforce the differences in locomotor activity between LPS-injected WT and KO mice (Fig. 7B–C). The recovery of the PKC δ KO mice from endotoxic shock was also readily apparent in the classic Rotarod test (Fig. 7D). The improved exploratory behavior observed in PKC δ knockouts is likely due to less LPS-induced septicemia in these mice or alternatively a faster recovery from septicemia. These results support a crucial role for PKC δ in regulating the systemic inflammatory response to LPS. We then determined the neuroinflammatory response in these mice as it relates to dopaminergic degeneration by studying the proinflammatory cytokine responses and microglial activation in the substantia nigra. Using qRT-PCR, we determined the changes in gene expression levels of the proinflammatory cytokine IL-12 in ventral midbrain tissue of LPS-injected PKC δ WT and KO mice. The gene expression studies were performed 48 h after the LPS injection, representing the time point when the PKC δ KO mice had recovered from LPS-induced motor deficits. Consistent with previous studies performed in an LPS neuroinflammation model (Qin et al., 2007), we observed a 40-fold increase in IL-12 mRNA levels in WT mice at 48 h post-LPS when compared to WT controls (Fig. 7E). Interestingly, in the ventral

midbrain of PKC δ KO mice, we found that IL-12 mRNA was restored to baseline values 48 h after systemic LPS challenge in contrast to WT mice. We recently reported that LPS-induced TNF α and IL-1 β were significantly reduced in PKC δ KO mice when compared to WT mice (Panicker et al., 2015). Together, these results suggest that PKC δ -mediated signaling is necessary for mediating LPS-induced proinflammatory cytokine response and associated behavioral deficits.

PKC δ knockout mice are resistant to MPTP-induced motor and behavioral deficits

Motor impairments arising from nigrostriatal dopaminergic neurodegeneration and dopamine deficits are hallmarks of PD in humans and have been recapitulated in the mouse MPTP model. To test whether PKC δ deficiency protects against neurobehavioral deficits in a MPTP mouse model of PD, we adopted the well-characterized acute MPTP mouse model (Hu et al., 2008; Kim et al., 2007; Przedborski et al., 2004; Wu et al., 2003) where both WT and PKC δ KO mice were treated with 4 doses of 18 mg/kg MPTP at 2-h intervals. Five days following the last dose of MPTP, WT and KO mice were tested for behavioral parameters and locomotor activity using the Versamax locomotor activity monitor and the Rotarod. Representative motor activity maps of movement of WT and PKC δ KO mice in the presence and absence of MPTP treatment are shown in Fig. 8A. Significant improvement in motor functions was obvious in the PKC δ KO mice treated with MPTP when compared to WT mice as seen with the improved behavioral parameters including vertical activity (Fig. 8B), horizontal activity (Fig. 8C), total distance traveled (Fig. 8D), total rest time (Fig. 8E) and total movement time (Fig. 8F). Improved motor functions were also apparent with the Rotarod test (Fig. 8G). Together, these results suggest that PKC δ deletion can rescue locomotor deficits in the MPTP mouse model of dopaminergic degeneration.

PKC δ knockout dramatically attenuates microglial activation and neuroinflammatory response in the MPTP model

To further determine if PKC δ signaling during microglial activation can regulate neuroinflammatory responses and drive dopaminergic degeneration *in vivo*, both WT and PKC δ KO mice were treated according to the acute MPTP protocol (4 doses of 18 mg/kg MPTP at 2-h intervals) and the neuroinflammatory response was measured 24 h later (Czlonkowska et al., 1996; Du et al., 2001; Przedborski et al., 2004; Wu et al., 2003). We first probed for microglial activation with the marker Iba1. MPTP induced a robust microglial activation response manifested by enlarged cell bodies, irregular shape and intensified Iba1 staining in WT mice (Fig. 9A, top panel), while MPTP-treated PKC δ KO mice exhibited less microglial activation morphology and a lower microglial cell density (Fig. 9A, lower panel), indicating that PKC δ is crucial for microglial activation following MPTP-induced neurotoxic insult. Next, we analyzed changes in the proinflammatory mediators that are known to be elevated in this model at around 24 h. MPTP upregulated the mRNA levels of TNF α , IL-1 β and iNOS as expected in WT mice. In KO mice, however, the MPTP-induced cytokine and iNOS production was greatly reduced (Fig. 9B–D), similar to the results obtained with LPS-treated mice (Fig. 7E). We further examined the levels of gp91phox, a subunit of the NADPH oxidase complex, which is a major source of ROS in the ventral midbrain regions of PD patients and in animal models of PD (Wu et al., 2003). Immunoblot analysis revealed that MPTP led to a robust increase in the nigrostriatal

gp91phox expression levels in WT mice. Interestingly, ablation of PKC δ completely suppressed gp91phox levels not only in basal conditions but also after MPTP treatment (Fig. 9E). Our findings are consistent with previous findings that PKC δ is involved in the phosphorylation of NADPH components (Bey et al., 2004; Fontayne et al., 2002; Khwaja and Tatton, 1999; Nitti et al., 2007). Moreover sequential activation of PKC δ and NOX2 was found to underlie the enhancement of ROS species in c6 glial cells exposed to paraquat, a dopaminergic neurotoxicant (Kim et al., 2008). Together, these data indicate that PKC δ is a critical mediator of not only microglial activation and the associated neuroinflammatory response, but also the induction of proinflammatory NADPH oxidase (gp91phox) in the SNpc of the MPTP model.

PKC δ deletion confers resistance against MPTP-induced striatal dopamine depletion and nigral TH neuronal loss

Microglial neuroinflammation and oxidative stress exacerbate dopaminergic degeneration in the nigrostriatal system. The reduced microglial activation and attenuation of neuroinflammatory factors in PKC δ KO mice both *in vitro* and *in vivo* prompted us to examine whether PKC δ KO mice are protected against nigrostriatal dopaminergic degeneration in the MPTP mouse model of PD. Mice were treated with MPTP (18 mg/kg) and 7 days later the brains were processed for quantification of the integrity of dopaminergic cell bodies and the projecting striatal dopaminergic fibers using TH-DAB immunostaining. The levels of TH-positive dopaminergic neurons in the substantia nigra of MPTP-treated WT mice had dramatically decreased (Fig. 10A). Similarly, a pronounced reduction of TH-positive terminal fibers had occurred in the striatum. Stereological counts of nigral dopaminergic neurons following MPTP treatment revealed that PKC δ KO mice had a remarkable preservation of TH positive neurons (Fig. 10B). Subsequently, to determine whether genetic depletion of PKC δ protects against neurochemical deficits caused by MPTP, we quantified tissue levels of striatal dopamine and its metabolites HVA and DOPAC by HPLC. As expected, MPTP caused a substantial loss of dopamine and its metabolites in the WT C57BL mice at 7 days when compared to saline-treated controls. In PKC δ KO mice, however, levels of dopamine and its metabolites were significantly preserved after MPTP treatment (Fig. 10C–E). Collectively, these results suggest that PKC δ deficiency is sufficient, at least in part, for preservation of the nigrostriatal dopaminergic system following exposure to the dopaminergic neurotoxin MPTP in mice.

Discussion

Multiple immunological stimuli have been shown to trigger microglial activation leading to sustained chronic inflammation in dopaminergic neurodegenerative processes (Hirsch et al., 2013; Hirsch et al., 2012; Rocha et al., 2015). In this study, we investigated the role of PKC δ signaling in regulating microglial activated-inflammatory responses and its relationship to dopaminergic neurotoxicity in both cell culture and murine models of PD. We provide evidence that activating microglia with diverse inflammogens, including TNF α , LPS, and aggregated α -synuclein, triggers the activation of PKC δ , which is accompanied by enhanced PKC δ gene expression and kinase activity, supporting the notion that increased PKC δ protein expression and associated kinase activity is integral to microglial activation.

Interestingly, a time-dependent increase in the nuclear translocation of PKC δ was also evidenced in microglia in response to diverse inflammatory challenges. Furthermore, PKC δ -deficient primary microglia exhibited diminished proinflammatory factor release, ROS and nitric oxide production following inflammogen challenges. Notably, we found that pharmacological inhibition or gene knockdown/deletion of PKC δ suppressed NF κ B activation, suggesting that NF κ B activation occurs via a PKC δ -dependent mechanism in response to microglial activation. We also extended our *in vitro* findings to *in vivo* experimental models of PD. In order to further validate our *in vitro* findings performed in cultured microglial cells, we tested the beneficial effects of PKC δ deficiency in *in vivo* models of neuroinflammation, namely LPS and MPTP mouse models. We demonstrated that PKC δ deficiency significantly reduced both the microglial activation and elevated proinflammatory factor production normally induced by LPS. Moreover, ablation of PKC δ ameliorated MPTP-induced loss of nigrostriatal dopaminergic neurons and associated neurobehavioral deficits when compared with drug-treated WT mice. Most importantly, we demonstrated the increased expression of PKC δ within Iba1-positive reactive microglia in the ventral mesencephalon of postmortem PD brains when compared to age-matched controls, thus validating the translational relevance of our results generated in both cell culture and mouse models of PD. Our recent study by Panicker et al. (Panicker et al., 2015) reported that PKC δ knockout mice displayed significantly attenuated LPS-induced cytokine production and 6-OHDA-induced nigrostriatal dopaminergic neuronal deficits and microgliosis. However, in that study we focused primarily on the functional role of Fyn kinase during microglial activation. Fyn kinase is upstream of PKC δ and can regulate PKC δ activation by tyrosine phosphorylation as we have previously shown (Saminathan et al., 2011). The current study focuses on the role of PKC δ in regulating microglial activation, especially whether the kinase is upregulated at both the mRNA and protein levels. As such, our results presented here provide new mechanistic insight into the molecular signaling pathways regulating microglia-mediated inflammation and its potential impact on dopaminergic degeneration. Taken together, our findings provide compelling evidence that PKC δ is a critical regulator of the proinflammatory response and that sustained activation of PKC δ contributes to PD pathogenesis by enhancing the microglia-mediated neuroinflammatory response.

The role of ROS in the regulation of signaling events in multiple cell types including microglia has been well established. In this context, activated microglia, in addition to its ability to generate cytokines and chemokines, is the major source for ROS in multiple neurodegenerative diseases (Block et al., 2007). Excessive free radicals generated from activated microglia following enzymatic activation of NADPH oxidase (PHOX) and iNOS have been identified as major contributors to the pathophysiology of numerous neurodegenerative diseases including PD (Brown and Neher, 2014). PKC δ mediates iNOS induction via ERK1/2 activation in IL-1 β -stimulated vascular smooth muscle cells (Ginnan et al., 2006), and it regulates iNOS induction following stimulation of BV-2 cells with LPS (Wen et al., 2011). On the other hand, NOX2 is highly expressed in innate immune cells including microglia, while NOX2 and NOX4 are expressed by neurons (Urrutia et al., 2014). Several stimuli including proinflammatory cytokines, LPS, and peroxynitrite have been shown to promote NOX activation. Furthermore, PKC activators are known to induce NOX

activation (Inoguchi et al., 2003). Therefore, we hypothesized that PKC δ -mediated upregulation of ROS is critically linked to oxidative stress and microglial activation following exposure to inflammogens. Our data show that PKC δ deficiency and shRNA-mediated gene silencing of PKC δ dramatically reduced inflammogen-mediated ROS generation and nitrite release in primary microglia cultures (Fig. 3). Our findings are consistent with previous studies in other models demonstrating that PKC δ is a critical regulator of iNOS expression in mouse peritoneal macrophages (Bhatt et al., 2010), human leukemia cells (Deb et al., 2003), and BV-2 cells (Wen et al., 2011). Likewise, in the SN of MPTP-treated PKC δ KO mice, a concomitant reduction of iNOS mRNA and gp91phox expression levels was detected in the nigral tissue at 24 h after MPTP treatment. Consistent with our findings, PHOX $-/-$ mice and mixed neuron-glia culture isolated from PHOX $-/-$ mice were both found to be more resistant to dopaminergic toxicity when compared to PHOX $+/+$ controls (Qin et al., 2004). Thus, these findings highlight the pivotal role of PKC δ in regulating iNOS induction and NOX activation in response to inflammatory stressors in primary microglial cells and in an MPTP mouse model of PD.

PKC δ is involved in numerous cellular functions, including apoptosis, cell survival, migration and proliferation (Wie et al., 2014). The unique capability of PKC δ to regulate numerous cellular functions is attributed, at least in part, to its differential subcellular localization, especially stimulus-dependent organelle translocation. Intriguingly, nuclear translocation of PKC δ has proven to be a critical determinant of cell fate (Humphries et al., 2008; Kanthasamy et al., 2003). For example, in response to apoptotic inducers and genotoxins, PKC δ is activated via phosphorylation at Tyr64 and Tyr155, which is requisite for its nuclear translocation (Humphries et al., 2008). Similarly, the nuclear translocation sequence (NLS) is essential for the induction of apoptosis owing to the activation of apoptotic substrates that are localized to the nucleus. In this context, Wallerstedt et al. (2010) demonstrated a direct correlation between nuclear translocation of PKC δ and induction of inflammatory genes in response to IL-6 in adipose cells. Consistent with their findings, the present report demonstrates that stimulation of microglia with diverse inflammogens including TNF, LPS, and α -synuclein increased PKC δ nuclear translocation within 30 min of stimulation and it remained elevated for a prolonged period of time (Fig. 2). Despite the nuclear translocation of PKC δ , we failed to see cell death in cells exposed to the inflammogens LPS and TNF α , suggesting that for a given stimulus and cell type, PKC δ activation might elicit a differential response and that nuclear translocation of PKC δ following microglial activation may not be required to induce an apoptotic response. Alternatively, it might promote the induction of NF κ B-dependent inflammatory downstream signaling events involving increased transcription of inflammatory factor production, ultimately leading to a heightened microglial activation response.

NF κ B is a key transcription factor involved in the regulation of inflammatory mediator production and immune cell activation in microglial cells (Hayden and Ghosh, 2004). NF κ B activation has been shown in the MPTP model of PD and in postmortem PD brains (Hunot et al., 1997). NF κ B activation is regulated by the activation of upstream IKKs, which in turn phosphorylates I κ B α , resulting in the subsequent proteasomal degradation of I κ B α and translocation of the p65 subunit to the nucleus where it promotes proinflammatory gene expression. Consistent with these studies, we observed that exposing primary microglial

cells to diverse inflammatory stimuli, including LPS and TNF α , resulted in a dramatic I κ B α degradation and NF κ B-p65 nuclear translocation (Fig. 5). Importantly, PKC δ deficiency in primary microglia remarkably abolished these events. Thus, PKC δ could participate in the positive regulation of NF κ B by promoting p65 transactivation. NF κ B controls the microglia-mediated inflammatory responses, but the signaling mechanisms underlying NF κ B activation and microglial activation during the dopaminergic neurodegenerative process is not well understood. In our study, p65 phosphorylation was enhanced in LPS- and TNF α -treated microglia, but importantly, this effect was abolished by genetic and siRNA-mediated knockdown of PKC δ . Consistent with our findings, Ren et al. (2014) showed that PKC δ interacts with cytosolic p65 and regulates PMA-induced chemokine expression in vascular smooth muscle cells by facilitating increased p65 phosphorylation and NF κ B transactivation. Given that NF κ B tightly regulates the expression of proinflammatory cytokines via ROS (Das et al., 1995), and that PKC δ gene knockdown reduced ROS generation following LPS-induced stimulation of microglial in the present study (Fig. 3E), it is plausible that ROS activates PKC δ , which in turn leads to p65 transactivation and enhanced transcription of proinflammatory mediators. Indeed, Vancurova et al. (2001) demonstrated that NF κ B activation is mediated via PKC δ -mediated I κ B α degradation in TNF α -stimulated neutrophils. Thus, it is likely that a positive feedback loop involving the PKC δ -NF κ B mechanism regulates, at least in part, the generation of proinflammatory mediators. Such a mechanism may effectively promote microglia-associated immune stimulation thereby leading to the progressive loss of nigrostriatal dopaminergic neurons.

Cytokines represent the major inflammatory mediators in the CNS that most often act through specific receptor-mediated mechanisms, thereby triggering the activation of downstream signaling cascades (Rocha et al., 2015). In the CNS, activated microglia produce numerous inflammatory cytokines including TNF α , IL-1 β , and IL-6, which play a central role in initiating the proinflammatory response (Sadasivan et al., 2012). The activation of NF κ B produces TNF α , IL-1 β , IL-6, IL-12, and IFN- γ in the microglia (Murakami and Hirano, 2012; Napetschnig and Wu, 2013). The persistent activation of microglia prolongs immune stimulation, thereby leading to the progressive neuronal damage implicated in neurodegenerative diseases. PKC isoforms also participate in the induction of an inflammatory response. For example, in murine macrophages, LPS-induced PKC α activation proved important for the regulation of TNF α and IL-1 β transcriptional machinery, while PKC β activation was critical for TNF α production (St-Denis et al., 1998). However, the precise role of PKC δ in regulating cytokine and chemokine production in activated microglia remains to be elucidated. In the present study, we show that wild-type primary microglia exerted a potent time-dependent increase in the generation of proinflammatory cytokines such as TNF α and IL-1 β in response to LPS and α -synuclein. In contrast, PKC δ KO microglia displayed a blunted activation response following exposure to these inflammogens. Likewise, increased generation of several inflammatory mediators such as chemokines and cytokines were evidenced at 24 h in microglia, yet this effect was effectively attenuated in the PKC δ KO microglia. Given that murine microglia express high levels of PKC δ relative to other isoforms (Wen et al., 2011), it is likely that PKC δ plays a key regulatory role in mediating an inflammatory response upon microglial activation. Together, these findings indicate that PKC δ is a major proinflammatory kinase that regulates

cytokine production in microglial cells and that blocking PKC δ activation dampens the microglial proinflammatory response and subsequent dopaminergic neurotoxicity.

Based on our *in vitro* data demonstrating that PKC δ -mediated microglial activation may in fact be a significant source of inflammatory mediators, we sought to confirm and extend our findings *in vivo*. For this purpose, we utilized two PD models, namely the LPS and MPTP models, to determine the exact contribution of PKC δ to the induction of neuroinflammatory responses by evaluating the microglial inflammatory response and its impact on nigrostriatal dopaminergic degeneration. We found that after systemic administration of LPS, PKC δ KO mice exhibited improved recovery from locomotor deficits when compared to WT littermates. Moreover, in PKC δ KO mice the amelioration of impaired behavior positively correlated with a marked attenuation of mRNA levels of proinflammatory cytokines. Consistent with our results, Hunter et al. (2007) demonstrated that intranigral LPS administration induces microglial activation and a proinflammatory response which was found to closely parallel the deleterious cascade of events leading to the death of dopaminergic neurons. Our overall results demonstrate a high degree of consistency showing that microglial activation and motor impairments are dramatically reduced in PKC δ KO mice when compared to WT mice following LPS treatment. Next, we investigated the effects of PKC δ gene ablation on MPTP-induced dopaminergic neurotoxicity and associated behavioral deficits. Our results showed a significant neuroprotective response against MPTP-induced neurotoxicity in PKC δ KO mice as revealed by the preservation of nigral dopaminergic neurons, the restoration of striatal dopamine levels relative to their WT counterparts, and consequently, the attenuation of drug-induced locomotor deficits and improved motor coordination. Our results agree with previous reports demonstrating that genetic knockdown of proinflammatory mediators and enzymes involved in the generation of RNS confers resistance against MPTP-induced neurotoxicity (Furuya et al., 2004; Liberatore et al., 1999). Currently, we are not aware of any specific abnormalities in our PKC δ knockout animals in terms of baseline microglial or neuronal cell numbers. However, we acknowledge that developmental abnormalities and compensatory mechanisms are potential issues when utilizing any strain of knockout mice. In our experience, the major abnormality we observed is a lower number of pups in PKC δ KO mice compared to wild type, suggesting a possible impairment in reproductive capacity. It is plausible that PKC δ activation in neurons leads to apoptosis while PKC δ activation in microglia contributes to a heightened microglial activation response. The robust protection of the nigrostriatal dopaminergic system evidenced in the PKC δ KO mice following exposure to LPS or MPTP may be attributed, at least in part, to the marked suppression of PKC δ -dependent processes, such as apoptosis in dopaminergic neurons (Anantharam et al., 2002; Kaul et al., 2003; Zhang et al., 2007a), microglial activation, or both. It should be noted that, without conditionally knocking out PKC δ in microglia, it is difficult to precisely define the role of microglial PKC δ signaling and its contribution to dopaminergic degeneration in the proposed experimental models. Furthermore, we previously reported that PKC δ signaling can negatively regulate the levels of tyrosine hydroxylase through its effects on protein phosphatase 2A, thereby influencing the levels of dopamine and DOPAC in dopaminergic terminals in the striatum of PKC δ knockout mice (Zhang et al., 2007b), compared with the present study where a slight decline in basal dopamine and DOPAC levels was evidenced.

The disparate nature of the data might at least in part be explained by the significant genetic drift due to genetic stress and/or inbreeding in small isolated populations, which may eventually account for divergent phenotypes in null-mutant colonies (Almodovar et al., 2013; Bijlsma et al., 1997; Brown et al., 2001; Swindell and Bouzat, 2006). Nonetheless, our results demonstrate that PKC δ knockout mice have reduced microglial activation *in vivo* in the CNS in response to a systemic inflammatory insult, highlighting the important role of this kinase in microglial activation. This is further supported by results from our mechanistic studies using isolated primary microglia isolated from PKC δ knockouts and wild-type microglia whereby shRNA-mediated knockdown of PKC δ expression markedly reduced the magnitude of the inflammatory response following exposure to LPS (Fig. 3). Collectively, our results suggest that PKC δ is critically linked to microglial activation and nigrostriatal dopaminergic neurodegeneration in both MPTP and LPS models.

In summary, gene ablation or shRNA-mediated gene silencing of PKC δ in the microglia resulted in the decreased generation of neurotoxic factors, attenuated NF κ B activation, and generation of proinflammatory mediators. PKC δ knockout mice exposed to LPS or MPTP displayed a phenotype consistent with reduced motor deficits in parallel with reduced microglial activation and preservation of nigrostriatal dopaminergic integrity. Our results support the hypothesis that increased PKC δ activation and an age-related elevation of the oxidative environment is conducive to maintaining a subpopulation of microglia in their proinflammatory phenotype, thereby exerting detrimental effects on dopaminergic neurons in PD brains. We conclude that the development of PKC δ -specific inhibitors represents a novel therapeutic intervention for neuroinflammation-related disorders including PD. Further studies should be aimed at investigating the neuroprotective efficacy of selective and brain-penetrating PKC δ inhibitors and related translational strategies in preclinical models of PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DCFH-DA	2',7'-dichlorodi-hydrofluorescein diacetate
FBS	fetal bovine serum
KO	knockout
TH	tyrosine hydroxylase
PKCδ	protein kinase C δ

PD	Parkinson's disease
PHOX	NADPH oxidase
WT	wild-type.

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Highlights

- Proinflammatory stressors including misfolded α -syn upregulates PKC δ in microglia
- PKC δ translocates to nucleus and promotes proinflammatory responses in microglia
- PKC δ mediates inflammagen-activated NF κ B signaling pathway in activated microglia
- PKC δ knockout mice exhibit dampened neuroinflammatory responses induced by LPS
- PKC δ deletion protects against neuroinflammation and dopaminergic neurodegeneration

Figure 1.

Increased PKCδ expression and kinase activity in inflammogen-activated microglia. **A**, PKCδ protein expression in primary microglia. Purified primary microglia were treated with LPS (100 ng/ml), TNFα (30 ng/ml), and aggregated α-synuclein (αSyn, 300 nM) for 12 and 24 h. The control group was treated with the vehicle (1x PBS) used to prepare LPS, TNFα and α-synuclein prior to treatment. PKCδ protein levels were probed by Western blotting using an antibody against the C-terminus of the protein that detects both the native and cleaved fragments. Equal loading was ascertained by tubulin. **B**, Increased PKCδ protein expression in inflammogen-activated BV-2 microglial cells. Immortalized BV-2 microglial cells were activated with LPS (1 μg/ml) and TNFα (30 ng/ml) for 24 h and then PKCδ protein levels were determined by Western blotting. β-actin was used as the loading control. Representative blots are shown and experiments were repeated at least three times. **C**, Increased PKCδ mRNA expression in primary microglia. SYBR Green real time quantitative PCR analysis for PKCδ mRNA expression in isolated primary microglial cells treated with LPS (100 ng/ml), TNFα (30 ng/ml) and aggregated α-synuclein (300 nM) for 24 h. The normalized fold increase in PKCδ gene expression over the control group was calculated by the C_t method. **D**, Increased PKCδ mRNA expression in BV-2 microglial cells treated with LPS (1 μg/ml) for 4, 8, 16 and 24 h. **E–F**, PKCδ kinase activity in activated microglia. The kinase activity of PKCδ was determined using a standard immunoprecipitation kinase assay. Primary microglia (**E**) and BV-2 microglial cells (**F**) were treated with either LPS (1 μg/ml), TNFα (30 ng/ml) or aggregated α-synuclein (300 nM) for 24 h. The PKCδ protein was immunoprecipitated from 500 μg of total protein and processed for the *in vitro* kinase activity assay using a histone substrate and radiolabeled [γ - 32 P] ATP. The kinase activity was quantified by densitometric analysis of the 32 P-histone

band intensity and expressed as percent control. The pure recombinant PKC δ protein was used as a positive control in primary microglia. Representative kinase assay gels are shown and kinase assays were repeated twice. **G**, Increased PKC δ mRNA expression in the MPTP-treated mouse ventral midbrain. **H**, Midbrain sections of PD patients and age-matched controls were double-labeled for Iba-1 and PKC δ . Results represent three independent experiments. Scale bar, 20 μ m. Data represented as the mean $\pm$ SEM for each group. Asterisks (***) $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$) indicate significant differences between control and treatment groups.

Figure 2.

Subcellular localization of PKC δ in inflammogen-activated microglial cells. **A–C**, Isolated primary microglia were treated with or without LPS (100 ng/ml), TNF α (30 ng/ml) and aggregated α -synuclein (300 nM) for 3, 12 and 24 h. Cells were fixed in 4% PFA and processed for immunofluorescence staining using a rabbit polyclonal antibody to PKC δ (Red, Alexa-555). Nuclei were labeled with Hoechst stain (blue). In microglia treated with LPS (**A**), TNF α (**B**) and aggregated α -synuclein (**C**), stimulus- and time-dependent nuclear translocation of PKC δ was visualized by confocal microscopy. **D–E**, Nuclear localization of PKC δ was determined by Western blotting the cytosolic and nuclear fractions prepared from primary microglia treated for 12 h with LPS (100 ng/ml), TNF α (30 ng/ml) and aggregated α -synuclein (300 nM). Equal loading was ascertained by Lamin B (nuclear fraction) and tubulin (cytosolic fraction). **F–G**, Subcellular localization of PKC δ in inflammogen-activated BV-2 cells. BV-2 cells were treated with LPS (1 μ g/ml, **J**) and TNF α (30 ng/ml, **K**) for 30 min, 1 and 3 h. Cells were fixed in 4% PFA and processed for immunofluorescence staining using a rabbit polyclonal antibody to PKC δ (Red, Alexa-555). Nuclei were labeled with Hoechst stain (blue). **H–K**, Time course of PKC δ nuclear localization was determined in the cytosolic and nuclear fractions prepared from BV-2 cells treated with LPS (1 μ g/ml, **H–I**) and TNF α (30 ng/ml, **J–K**) for 1 and 3 h using Western blot analysis. Equal loading was ascertained by Lamin B and tubulin.

Figure 3.

Lentiviral shRNA-mediated knockdown and genetic depletion of PKCδ attenuates LPS-induced microglial activation. **A**, Isolated primary microglia were transduced with lentiviral particles expressing either PKCδ shRNA or non-specific (NS) shRNA. The infection efficiency was determined by visualizing the GFP-tag co-expressed by the viral particles, and knockdown of the PKCδ native protein level was confirmed by Western blotting. **B**, LPS-induced nitric oxide production in shRNA-infected microglia. Primary microglia were infected with either non-specific or PKCδ shRNA virus and then treated with LPS (100 ng/ml) for 24 h. Nitric oxide levels in the supernatant were determined using the Griess reagent. PKCδ shRNA-infected primary microglial showed reduced nitrite levels in the supernatant compared to non-specific shRNA-infected microglia. **C**, Effect of PKCδ knockdown on LPS-induced proinflammatory cytokine production in primary microglia. Primary microglia were infected with either non-specific or PKCδ shRNA virus and treated with LPS (100 ng/ml) for 24 h, and then proinflammatory cytokines were quantified using the Luminex immunoassay system. Knockdown of PKCδ attenuated LPS-induced IL-6 and TNFα production but not IL-12 levels in the same supernatants. **D**, Nitric oxide production in primary microglial supernatants. Primary microglia were isolated from PKCδ wild-type (+/+) and knockout (-/-) mice treated with LPS (100 ng/ml) for 24 h, and nitric oxide production was determined indirectly by quantifying the nitrite levels in the supernatant using the Greiss reagent. Microglia isolated from PKCδ -/- mice showed significantly reduced nitric oxide production compared to +/+ microglial cells. **E-F**, Intracellular ROS generation in microglia. Primary microglia from PKCδ +/+ and -/- mice were treated with LPS (100 ng/ml, E) and TNFα (30 ng/ml, F) for 6 h and intracellular ROS generation was quantified using the fluorescent DCFDA dye. Microglia isolated from PKCδ -/- mice had

attenuated intracellular ROS levels compared to those isolated from PKC δ $+/+$ mice when stimulated with either LPS or TNF α . No significant changes in ROS levels were observed in PKC δ $+/+$ and $-/-$ mouse microglia stimulated with 300 nM aggregated α -synuclein (data not shown). Data represented as the group mean $\pm$ SEM ($n = 4$ to 8 per group). Experiments were repeated three times. Asterisks (***) $p < 0.001$ and ** $p < 0.01$ indicate significant differences between LPS-treated groups or TNF α -treated groups and “ns” denotes no significant difference.

Figure 4.

Reduced cytokine and chemokine production in microglia isolated from PKCδ knockout mice in response to inflammogen stimulation. Primary microglia from PKCδ wild-type (+/+) and knockout (-/-) mice were treated with LPS (100 ng/ml, A–B) and aggregated α-synuclein (300 nM, C–E) for 24 h. Cytokine levels in the supernatant were quantified at various time points (8, 16 and 24 h) using the Luminex immunoassay system. Data represented as the group mean ± SEM (n = 4–6 per group) from two experiments run on a 32-plex assay system. Asterisks (***) $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ indicate significant differences between LPS-treated groups or α-synuclein-treated groups from PKCδ +/+ and PKCδ -/- microglial cells.

Figure 5.

PKCδ regulates inflammogen-induced activation of the NFκB signaling pathway in activated microglia. **A–B**, LPS- and TNFα-induced IκBα degradation in primary microglia is suppressed in PKCδ knockout microglia. Wild-type (+/+) and PKCδ knockout (-/-) microglia were treated with LPS (100 ng/ml) or TNFα (30 ng/ml). Whole-cell lysates were probed with IκBα antibody using Western blot, with equal loading ascertained by tubulin. Asterisks (***) $p < 0.001$ indicate significant differences between LPS-treated groups or TNFα-treated groups from PKCδ +/+ and PKCδ -/- microglial cells. **C–D**, siRNA-mediated PKCδ knockdown suppressed LPS-induced IκBα degradation in primary microglia. For PKCδ knockdown studies, primary microglia were transfected with either scrambled or PKCδ-specific siRNA for 48 h. Transfected microglia were subsequently treated with or without LPS (100 ng/ml) for 15–60 mins. Whole-cell lysates were probed with IκBα antibody using Western blot, with equal loading ascertained by tubulin. Asterisks indicate significant differences between the respective treatment groups (** $p < 0.01$ vs LPS-treated scrambled siRNA-transfected cells; # $p < 0.01$ vs untreated scrambled siRNA-transfected cells). **E–F**, PKCδ +/+ and PKCδ -/- microglia were treated with or without LPS (100 ng/ml, E) and TNFα (30 ng/ml, F) for 15 minutes. For ICC studies, cells were fixed in 4% PFA and processed for immunofluorescence staining/confocal microscopy using a rabbit polyclonal antibody to p65 (Green). Nuclei were labeled with Hoechst stain (Blue). **G**, Non-specific siRNA- and PKCδ-specific siRNA-transfected primary microglia were treated with or without LPS (100 ng/ml) for 15–60 min. Nuclear and cytosolic extraction was then carried out and p65 nuclear translocation was quantified by Western blot analysis in both cytosolic and nuclear lysates. Lamin B and tubulin were used as loading controls for nuclear and cytosolic fractions, respectively.

Figure 6.

PKC δ regulates inflammogen-induced p65 phosphorylation in activated microglia. **A–B**, LPS- and TNF α -induced p65 phosphorylation at Serine 536 in primary microglia was suppressed in PKC δ knockout microglia. Wild-type (+/+) and PKC δ knockout (-/-) microglia were treated with or without LPS (100 ng/ml) and TNF α (30 ng/ml) for. Whole-cell lysates were probed with a phospho-p65 antibody using Western blot, with equal loading ascertained by tubulin. Asterisks (* $p < 0.05$) indicate significant differences between LPS-treated groups or TNF α -treated groups from PKC δ +/+ and PKC δ -/- microglial cells. **C–E**, siRNA-mediated PKC δ knockdown (C–D) and PKC δ inhibitor, rottlerin (E), suppressed LPS-induced p65 phosphorylation in primary microglia. For knockdown studies, primary microglia were transfected with non-specific and PKC δ -specific siRNA for 48 h and then treated with or without LPS (100 ng/ml) for 15 min. For PKC δ inhibitor studies, microglia were pre-incubated for 1 h with rottlerin (2 μ M) or DMSO and then stimulated with 100 ng/ml LPS for 5–30 min. Whole-cell lysates were probed with a phospho-p65 antibody using Western blot, with equal loading ascertained by β -actin or tubulin. Asterisks indicate significant differences between the respective treatment groups (** $p < 0.01$ vs LPS-treated scrambled siRNA-transfected cells; # $p < 0.05$, ## $p < 0.01$ vs untreated scrambled siRNA-transfected cells).

Figure 7.

PKC δ knockout mice have reduced nigral proinflammatory cytokines and are resistant to LPS-induced endotoxemia. **A**, Representative maps (VersaPlot) of vertical rearing activity (red circles) and horizontal movement tracks of saline- and LPS-injected mice. Age-matched PKC δ WT (+/+) and KO (-/-) mice were injected with a single dose of LPS (5 mg/kg, i.p.) or equivalent volumes of saline and their behavioral activity was assessed 48 h later using the VersaMax Analyzer. LPS-injected PKC δ -/- mice exhibit near normal locomotor activity similar to saline-injected controls (lower panel). In comparison, +/+ mice showed typical symptoms of endotoxemia and lethargic movement (top panel). **B**, Total horizontal movements and **C**, Total vertical activity of saline- and LPS-injected mice quantified using the VersaMax Analyzer at 48 h showing significantly improved activity in PKC δ -/-. **D**, Rotarod performance. At 48 h after LPS (5 mg/kg) treatment, LPS-injected PKC δ -/- mice spent more time on the rotarod compared to +/+ mice. Data represented as the group mean $\pm$ SEM from 6 to 8 mice. Experiments were repeated three times. Asterisks (***) $p < 0.001$ and ** $p < 0.01$ indicate significant differences between LPS-treated groups from PKC δ +/+ and PKC δ -/- mice. ## denotes $p < 0.001$ compared to saline-injected mice. **E**, Real-time SYBR green qRT-PCR for nigral pro-inflammatory cytokines. PKC δ +/+ and -/- mice were injected with 5 mg/kg LPS (ip) and proinflammatory cytokine gene expression was determined by real-time PCR at 48 h. Levels of IL-12 were elevated following LPS stimulation in PKC δ +/+ mice but were significantly reduced in PKC δ -/- mice. Data represented as the group mean $\pm$ SEM ($n = 4$ mice per group). Experiments were repeated at least three times. Asterisks (** $p < 0.01$) indicate significant differences between LPS-treated groups from PKC δ +/+ and PKC δ -/- mice. # $p < 0.01$ denotes significant differences between LPS- and saline-treated +/+ groups.

Figure 8.

PKC δ knockout mice are protected against MPTP-induced locomotor deficits. **A**, VersaPlot movement track of mice. Locomotor activity was analyzed 5 days after MPTP treatment in PKC δ wild-type (+/+) and knockout (-/-) mice using VersaMax Analyzer. Representative horizontal movement tracks (VersaPlot) and vertical rearing events (red circles) of the mice collected over 10 min were plotted for a single mouse from each group. **B–F**, Quantification of locomotor activity using the VersaMax Analyzer. Saline- or MPTP-treated mice were placed in the VersaMax monitor for 10 min and various locomotor activity parameters were measured simultaneously. PKC δ -/- mice showed significantly improved vertical activity (**B**), horizontal activity (**C**), total distance travelled (**D**), total rest time (**E**) and total movement time (**F**). **G**, Rotarod performance analysis was performed 5 days after MPTP treatment and the amount of time spent on the rotarod was measured in PKC δ +/+ and -/- mice. Data represented as the mean $\pm$ SEM of six mice per group. Asterisks (** $p < 0.01$ and * $p < 0.05$) denote significant differences between MPTP-treated +/+ and PKC δ -/- mice; # $p < 0.01$ and ## $p < 0.001$ denote significant differences between saline- and MPTP-treated +/+ groups. “ns”, not significant.

Figure 9.

PKCδ knockout mice have reduced microglial activation and proinflammatory responses in the MPTP model of dopaminergic degeneration. **A**, Immunocytochemistry for Iba1-positive microglia in nigral brain sections. Ventral midbrain sections from 24-h MPTP-treated PKCδ wild-type (+/+) and knockout (-/-) mice were stained for the microglial marker Iba1 using DAB immunohistochemistry. Images were captured at 10× and 20× (inserts) magnification. The nigral zone is outlined for each field (white ellipses). Extensive microglial activation is evident in MPTP-treated +/+ mice whereas PKCδ -/- mice had reduced Iba1 staining. **B–D**, Quantitative real-time PCR for pro-inflammatory cytokines in MPTP-treated nigral tissues. Age-matched PKCδ +/+ and -/- mice were injected with 4 doses of MPTP (18 mg/kg) or saline at 2-h intervals. Nigral tissues were dissected 24 h after the last injection and mRNA levels for TNFα, IL-1β and iNOS were determined by real-time SYBR green quantitative RT-PCR. The 18S rRNA gene was used as the internal control and the data are expressed as fold change over the saline-treated group. Data represented as the group mean ± SEM (n = 4 mice per group). Experiments were repeated twice. **E**, Western blot for gp-91phox. Nigral tissues from PKCδ +/+ and -/- mice were dissected 24 h after the last injection of either saline (S) or MPTP (M). Lysates were probed by Western blotting for gp91phox, the membrane-bound subunit of the NADPH-oxidase complex, using a mouse monoclonal antibody; β-actin was used as a loading control. A representative blot is shown and experiments were repeated three times. Asterisks (* $p < 0.05$ and ** $p < 0.01$) indicate significant differences between MPTP-treated groups from PKCδ +/+ and PKCδ -/- mice. ## $p < 0.001$ denotes significant differences between saline- and MPTP-treated +/+ groups.

Figure 10.

PKC δ knockout mice are protected against MPTP-induced nigrostriatal dopaminergic degeneration. **A**, DAB immunohistochemistry for tyrosine hydroxylase (TH) in substantia nigral and striatal sections. Mice were perfused 7 days after MPTP dosing, 30- μ m sections were processed for TH-DAB immunostaining and SNpc images were captured at 2 $\times$ and 20 $\times$ (middle panel) magnification. Striatal sections were imaged at 2X magnification. Representative images are shown and experiments were repeated three times. **B**, Stereological counting of TH-positive neurons in the substantia nigra. The number of surviving TH-positive dopaminergic neurons in MPTP-treated PKC δ wild-type (+/+) and knockout (-/-) mice at 7 days was counted using unbiased stereological analysis as described in Methods. After MPTP treatment, PKC δ -/- mice had a significantly more TH-positive dopaminergic neurons than did +/+ mice. **C-E**, HPLC analysis of striatal dopamine and its metabolites. PKC δ +/+ and -/- mice were treated with 4 doses of MPTP (18 mg/kg) or saline at 2-h intervals and were sacrificed 7 days after the last injection. The levels of striatal dopamine and its metabolites DOPAC and HVA were determined by HPLC. After MPTP treatment, PKC δ -/- mice had significantly higher levels of dopamine, DOPAC and HVA. Data represented as the mean $\pm$ SEM of 6 to 7 mice per group. Asterisks indicate significant differences between the respective treatment groups (* $p < 0.05$ and *** $p < 0.001$ vs +/+ MPTP; ## $p < 0.001$ vs +/+ Control).

Table 1

Primary microglia were isolated from PKC δ wild-type (+/+) and knockout (-/-) mice and treated with LPS (100 ng/ml) or TNF α (30 ng/ml) for 24 h. Cytokine and chemokine levels in the supernatant were quantified using the Luminex immunoassay system. Data represented as the group mean $\pm$ SEM from three to four experiments.

Reduced cytokine and chemokine production in microglia isolated from PKC δ knockout mice in response to LPS and TNF α stimulation

<i>Cyto/Chemokine</i>	PKC $\delta^{+/+}$ (pg/ml)			PKC $\delta^{-/-}$ (pg/ml)		
	Control	LPS	TNF α	Control	LPS	TNF α
IL-1 α	0.58 $\pm$ 0	274.77 $\pm$ 27.17 ^{##}	ND	0.58 $\pm$ 0	202.38 $\pm$ 27.25 ^{**}	ND
IL-6	14.56 $\pm$ 4.49	ND	217.21 $\pm$ 33.57 ^{##}	3.38 $\pm$ 0	ND	27.59 $\pm$ 11.75 ^{***}
IP-10	244.72 $\pm$ 13.61	2176.35 $\pm$ 67.34 ^{##}	652.26 $\pm$ 62.53 ^{##}	123.24 $\pm$ 32.26	893.00 $\pm$ 70.66 ^{***}	176.76 $\pm$ 59.03 ^{***}
KC	89.30 $\pm$ 15.54	10188.26 $\pm$ 522.76 ^{##}	1547.43 $\pm$ 108.51 ^{##}	25.73 $\pm$ 18.52	3776.53 $\pm$ 525.96 ^{**}	181.38 $\pm$ 50.82 ^{***}
MIP-2	22.71 $\pm$ 32.61	14034.23 $\pm$ 1884.31 ^{##}	534.06 $\pm$ 23.36 ^{##}	17.64 $\pm$ 22.48	9901.30 $\pm$ 865.16 ^{**}	234.77 $\pm$ 11.65 ^{***}
MIP-1 α	136.09 $\pm$ 16.91	5775.25 $\pm$ 378.32 ^{##}	207.84 $\pm$ 31.24 ^{##}	39.03 $\pm$ 12.45	973.29 $\pm$ 187.21 ^{***}	301.80 $\pm$ 70.07 [*]
MIP-1 β	113.25 $\pm$ 11.51	ND	213.89 $\pm$ 25.76 [#]	115.03 $\pm$ 20.50	ND	297.67 $\pm$ 43.55 ^{**}
MCP-1	136.09 $\pm$ 16.91	ND	2552.63 $\pm$ 111.57 ^{##}	39.03 $\pm$ 12.45	ND	280.55 $\pm$ 54.13 ^{***}
g-CSF	6.35 $\pm$ 0.10	ND	47.83 $\pm$ 6.93 ^{##}	6.40 $\pm$ 0	ND	20.83 $\pm$ 6.48 ^{***}

Asterisks (***) $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ indicate significant differences between LPS-treated groups from PKC δ WT and PKC δ knockout microglia; # $p < 0.01$ and ## $p < 0.001$ denote a significant difference between wild-type treatment groups and untreated controls. "ND", not determined.