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PKC δ knockout mice are protected from para-methoxymethamphetamine-induced mitochondrial stress and associated neurotoxicity in the striatum of mice

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Abstract

Para-methoxymethamphetamine (PMMA) is a para-ring-substituted amphetamine derivative sold worldwide as an illegal psychotropic drug. Although PMMA use has been reported to lead to severe intoxication and even death, little is known about the mechanism(s) by which PMMA exerts its neurotoxic effects. Here we found that PMMA treatment resulted in phosphorylation of protein kinase C δ (PKC δ) and subsequent mitochondrial translocation of cleaved PKC δ . PMMA-induced oxidative stress was more pronounced in mitochondria than in the cytosol. Moreover, treatment with PMMA consistently resulted in significant reductions in mitochondrial membrane potential, mitochondrial complex I activity, and mitochondrial Mn superoxide dismutase-immunoreactivity. In contrast, PMMA treatment led to a significant increase in intramitochondrial

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There is no conflict of interest

Ca²⁺ level. Treatment with PMMA also significantly increased ionized calcium binding adaptor molecule 1 (Iba-1)-labeled microglial activation and upregulated tumor necrosis factor alpha (TNF- α) gene expression. PKC δ knockout attenuated these mitochondrial effects and dampened the neurotoxic effects of PMMA. Importantly, TNF- α knockout mice were significantly protected from PMMA-induced increases in phospho-PKC δ expression, mitochondrial translocation of cleaved PKC δ , and Iba-1-labeled microgliosis. Both rottlerin, a pharmacological inhibitor of PKC δ , and etanercept, a pharmacological inhibitor of TNF- α , significantly protected against PMMA-mediated induction of apoptosis, as assessed by terminal deoxynucleotidyl transferase dUDP nick end labeling (TUNEL) assays. In addition, PKC δ knockout and TNF- α knockout both resulted in decreased PMMA-mediated induction of dopaminergic loss. Therefore, our results suggest that PKC δ mediates PMMA-induced neurotoxicity by facilitating oxidative stress (mitochondria > cytosol), mitochondrial dysfunction, microglial activation, and pro-apoptotic signaling. Our results also indicate that PMMA-induced PKC δ activation requires the proinflammatory cytokine TNF- α .

Graphical Abstract

Keywords

Para-methoxymethamphetamine; Protein kinase C δ ; Oxidative stress; Mitochondria; Tumor necrosis factor- α ; Microglia

1. Introduction

Methoxy-derivatives of phenylethylamines such as 3,4-methylenedioxymphetamine (MDA), 3,4-methylenedioxyethylamphetamine (MDMA), and paramethoxymethamphetamine (PMMA) exhibit hallucinogenic properties. Although PMMA has stimulant activity comparable to MDMA (McCreary et al., 1999; Bankson and Cunningham, 2002; Bubar et al., 2004; Freezer et al., 2005), PMMA is a weaker stimulant than methamphetamine (MA) (Fukushima et al., 2007; Hall et al., 2008; Schutova et al., 2009). The occurrence of stereotypical behavior is also comparable upon administration of

MDMA, stimulants, or hallucinogenics (Gold et al., 1989; Paulus and Geyer, 1992; Freezer et al., 2005; Palenicek et al., 2008). However, para-methoxy derivatives act more slowly and exhibit higher toxicity compared with their methylenedioxy counterparts (Schifano, 2004). More than 90 fatalities and many severe poisonings have been attributed to PMMA ingestion in Canada, the United States, Australia, and Europe (Hans, 2003; Johansen et al., 2003; Lin et al., 2007). Although PMMA has been shown to act in rats by a primarily serotonergic neurotoxic mechanism (Steele et al., 1992), we showed in a pilot study that PMMA appears to act on the dopaminergic system in mice, and that PMMA exerts neurotoxic effects (Nam et al., 2015). However, little is known about the mechanism(s) by which PMMA exerts these effects.

The protein kinase C (PKC) family consists of serine/threonine kinases that are broadly classified into three subgroups based on their sensitivity to the important cofactors, phospholipids and Ca^{2+} (Dempsey et al., 2000; Gschwendt, 1999). Conventional PKC isoforms (α , β I, β II, and γ) are sensitive to Ca^{2+} and diacylglycerol, whereas the novel isoforms (δ , ϵ , θ , η , and ζ) are Ca^{2+} -independent, but require diacylglycerol for activation. The atypical isoforms (ξ and ι/λ) require neither Ca^{2+} nor diacylglycerol for activation.

PKC isoforms are distributed differentially in tissues and play key roles in various cellular biological processes, including cell differentiation, cell growth, apoptosis, tumor suppression, and carcinogenesis. PKC inhibitors have typically been used to demonstrate the anti-apoptotic role of PKCs. PKC δ was the first novel isoform found to be functionally modulated by tyrosine phosphorylation upon H_2O_2 treatment (Konishi et al., 1997; Steinberg, 2004). An earlier report suggested that protein kinase C δ (PKC δ) plays a key role in apoptotic neuronal cell death (Kanthasamy et al., 2003).

Earlier studies have shown that PKC δ is proteolytically activated by caspase-3 during dopaminergic cell death, and that genetic or pharmacological targeting of PKC δ can protect against dopaminergic degeneration (Kaul et al., 2003; Zhang et al., 2007). In this paradigm, increased oxidative stress in dopaminergic neurons results in caspase-3-mediated proteolytic activation of PKC δ downstream of the intrinsic mitochondrial apoptotic pathway. The proteolytic cleavage of PKC δ by caspase-3 results in persistent activation. PKC δ is known to be an effector of dopaminergic cell death, and we recently demonstrated that PKC δ mediates methamphetamine-induced neurotoxicity (Dang et al., 2016; Narita et al., 2008; Nguyen et al., 2015; Shin et al., 2012 and 2014).

Therefore, we aimed to examine whether PMMA modulates a specific PKC isoform and whether PMMA exerts neurotoxic effects in mice. We observed that treatment with PMMA resulted in phosphorylation and mitochondrial translocation of PKC δ in the mouse striatum; that PKC δ mediates PMMA neurotoxicity by activating mitochondrial oxidative damage, mitochondrial dysfunction, microgliosis and proapoptotic signaling; and that crosstalk between PKC δ and tumor necrosis factor α (TNF- α) is also critical for PMMA-induced neurotoxicity. Since PMMA-induced dopaminergic toxicity was observed only in the striatum (Supplemental Figs. 1 and 3), we chose to focus on the striatum for this study.

2. Materials and Methods

2.1. Animals

All animals were treated in accordance with the National Institutes of Health (NIH) Guide for the Humane Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1985; www.dels.nas.edu/ila). The present study was performed in accordance with the Institute for Laboratory Research (ILAR) Guidelines for the Care and Use of Laboratory Animals. Mice were maintained under a 12-h light:12-h dark cycle and fed ad libitum. They were adapted to these conditions for 2 weeks prior to the experiment.

A breeding pair of PKC δ heterozygous knockout mice, originally bred into a C57BL/6 background, was a kind gift from Dr. K.I. Nakayama (Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) (Miyamoto et al., 2002). These mice were subsequently maintained and bred into the C57BL/6 background for three to six generations in an SPF mice facility before use with PKC δ WT mice from the same litter.

The tumor necrosis factor alpha (TNF- α) knockout mouse strain in the C57BL/6 background strain was previously described (Tran et al., 2012; Kim et al., 2013). Breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

To evaluate the effects of rottlerin and PKC δ antisense oligonucleotides on PMMA-mediated induction of apoptosis, we employed 10-week-old male ICR mice (Taconic Farms, Inc., Samtako Bio Korea, O-San, South Korea). We used these mice because we previously showed (Nguyen et al., 2015; Shin et al., 2012 and 2014) that methamphetamine increases the number of terminal deoxynucleotidyl transferase dUDP nick end labeling (TUNEL)-positive cells in the striatum of Taconic ICR mice, but not in the striatum of C57BL/6 mice.

2.2. Characterization of PKC δ knockout mice

Tail DNA was evaluated and typed by polymerase chain reaction (PCR). Primers for PKC δ amplification were obtained from Bioneer Corporation (Daejeon, South Korea). The PCR primers for genotyping were as follows: 5'-GGAAGAATAAGAACTGCATCACC-5' and 5'-GAAGGAGCCAGAACCGAAAG-3' for detection of the endogenous allele and 5'-GGAAGAATAAGAACTGCATCACC-3' and 5'-TGGGGTGGGATTAGATAAATG-3' for detection of the mutant allele. Brain tissue homogenates from PKC δ knockout (KO) mice were analyzed by Western blotting using antibodies against PKC δ and other isoforms thereof (α , β I, β II, ζ ; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). This analysis confirmed that PKC δ was selectively absent in PKC δ KO mice, and that the expression of the other isoforms was normal.

2.3. Synthesis of para-methoxymethamphetamine (PMMA)

Synthesis and use of PMMA for the present study were approved by the Korea Food and Drug Administration (KFDA), and were performed in strict accordance with KFDA regulations. Para-methoxymethamphetamine (PMMA) was synthesized as described previously (Montgomery et al., 2007) and its structure and purity were confirmed by the

following spectroscopic and HPLC analyses. ^1H NMR (400 MHz, CDCl_3 , δ): 7.13 (d, J = 8.7 Hz, 2H), 6.85 (d, J = 8.7 Hz, 2H), 3.79 (s, 3H), 3.40 (dd, J = 13.2 Hz, 3.9 Hz, 1H), 3.22–3.32 (m, 1H), 2.79 (dd, J = 13.2 Hz, 10.3 Hz, 1H), 2.69 (s, 3H), 1.34 (d, J = 6.3 Hz 3H); ^{13}C NMR (100 MHz, CD_3OD , δ) 159.3, 130.3 (2C), 127.7, 114.2 (2C), 56.8, 54.6, 38.2, 29.8, 14.5; HR-MS calcd for $\text{C}_{11}\text{H}_{18}\text{NO}$ $[\text{M}-\text{Cl}]^+$ 180.1383, found 180.1466; HPLC purity = 99.78%, Hypersil Gold C_{18} 5 μm (100×4.6 mm), mobile phase: 0.1% H_3PO_4 in water(A)/acetonitrile(B), 0–9 min (A/B = 90/10) and 9–11 min (A/B = 50/50).

2.4. Drug treatment

Wild-type (WT) and PKC δ KO mice were treated with PMMA (60 mg/kg, i.p.) or saline twice a day for 4 consecutive days and sacrificed at 1 h, 12 h, 1 d, and 7 d after the final PMMA injection. No WT mice died as a result of PMMA administration (20, 40, or 60 mg/kg, i.p.) in our experimental design. However, treatment with PMMA (80 mg/kg, i.p.) resulted in a 40 % mortality rate (4 of 10 mice) within 2 days in our experimental schedule, indicating that PMMA induces mortality in a dose-dependent manner. Furthermore, PMMA treatment (20 or 40 mg/kg, i.p.) did not induce significant dopaminergic loss (Nam et al., 2015).

We assessed the parameters of PKC δ phosphorylation; mitochondrial translocation of cleaved PKC δ ; protein oxidation; lipid peroxidation; ROS formation; mitochondrial membrane potential; intramitochondrial Ca^{2+} level; complex I activity; changes in tyrosine hydroxylase (TH), ionized calcium binding adaptor molecule 1 (Iba-1), mitochondrial manganese-dependent superoxide dismutase (MnSOD), PKC α , PKC β I, PKC β II, PKC ζ , PKC δ , TNF- α , tumor necrosis factor receptor 1 (TNFR1), tumor necrosis factor receptor 2 (TNFR2), interleukin-6 (IL-6), and interferon gamma (IFN- γ)- expression; dopamine level; and dopamine metabolite levels.

Taconic ICR mice were treated with a single dose of PMMA (80 mg/kg, i.p.) or saline and sacrificed 12 h later for quantification of PMMA-induced TUNEL-positive cells in the striatum. Rottlerin (PKC δ inhibitor; Biomol Research Laboratories Inc., Plymouth, PA, USA) was dissolved in dimethyl sulfoxide (DMSO) to form a stock solution, which was then stored at -20°C . The rottlerin stock solution was diluted in sterile saline to a concentration of 1 $\mu\text{g}/\mu\text{L}$ immediately before use. The final DMSO concentration was 10% (v/v). For intracerebroventricular (i.c.v.) infusion of etanercept (TNF- α inhibitor; Wyeth, Solna, Sweden), etanercept was diluted 1:10 in saline. Either rottlerin (3 $\mu\text{g}/3 \mu\text{L}$, i.c.v.) or etanercept (50 $\mu\text{g}/3 \mu\text{L}$, i.c.v.) was given at 4 h and 0.5 h, respectively, before PMMA injection. We based the dose of rottlerin on previous studies by our group (Nguyen et al., 2015; Shin et al., 2012 and 2014) and others (Nilsberth et al., 2009).

2.5. Guide cannula implantation and intracerebroventricular infusion

A stainless steel guide cannula (AG-4; Eicom, Kyoto, Japan) was implanted into the right lateral ventricle (stereotaxic coordinates: 0.5 mm posterior to bregma, 1 mm right of the midline, and 2 mm ventral to the dura) as described previously (Nguyen et al., 2015; Shin et al., 2011 and 2014). Microinfusion into the lateral ventricle was performed through a microinfusion cannula (AMI-4, Eicom) at a rate of 1 $\mu\text{L}/\text{min}$ using a microinjection pump

(CMA/100, CMA, Solna, Sweden). The microinfusion cannula was kept in place for 1 min after infusion to avoid backflow.

2.6. Preparation of cytosolic and mitochondrial fractions for Western blotting and neurochemical analyses

Cytosolic and mitochondrial fractions were prepared as previously described (Nguyen et al., 2015; Shin et al., 2014). Briefly, striatal tissues were collected and homogenized in ice-cold homogenization buffer containing 0.25 M sucrose, 0.5 mM potassium ethylene glycolbis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM Tris-HCl (pH 7.4), and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) using a Dounce homogenizer. Homogenates were centrifuged at $2,000 \times g$ for 10 min to remove nuclei and unbroken cells. The clarified supernatants were then centrifuged at $12,000 \times g$ for 15 min to obtain crude mitochondrial pellets and cytosolic supernatants. Crude mitochondrial pellets were suspended in 3% Ficoll 400 (Sigma-Aldrich) in Ficoll dilution buffer containing 0.25 M mannitol, 60 mM sucrose, 0.1 mM potassium EGTA, and 10 mM Tris-HCl (pH 7.4). A Ficoll density gradient was constructed by pouring the crude mitochondrial suspension (in 3% Ficoll) over a 6% Ficoll 400 solution. Purified mitochondrial pellets were obtained by centrifugation at $11,500 \times g$ for 10 min and then resuspended in buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and a protease cocktail (pH 7.4). For Western blotting, mitochondrial pellets were lysed in 100 μ L of lysis buffer.

2.7. Western blot analysis

Striatal tissues were lysed in buffer containing 200 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM EGTA, 5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 $\times$ phosphatase inhibitor cocktail I (Sigma-Aldrich), and 1 $\times$ protease inhibitor cocktail (Sigma-Aldrich). The resultant lysates were centrifuged at $12,000 \times g$ for 30 min, and the supernatant fractions were analyzed by Western blotting as previously described (Nguyen et al., 2015; Shin et al., 2012 and 2014). Proteins (20 μ g/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) on 8% or 10% gels and then transferred onto polyvinylidene fluoride (PVDF) membranes. Following transfer, the membranes were preincubated with 5% non-fat milk for 30 min and then incubated overnight at 4°C with primary antibodies against PKC α (1:70000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PKC β I (1:70000; Santa Cruz Biotechnology), PKC β II (1:5000; Santa Cruz Biotechnology), PKC ζ (1:10000; Santa Cruz Biotechnology), PKC δ (1:10000; Santa Cruz Biotechnology), Tyr311 p-PKC δ (1:500; Santa Cruz Biotechnology), cleaved PKC δ (1:2000; Santa Cruz Biotechnology), Iba-1 (1:500, Abcam, Cambridge, UK), MnSOD [1:10000; a kind gift from Dr. Kanefusa Kato, Aichi Prefectural Colony, Kasugai, Japan (Kim et al., 2000 and 2003; Kurobe and Kato, 1991)], IL-6 (1:1000; Abcam, Cambridge, UK), IFN- γ (1:5000; Bio-Rad Laboratories, CA, USA), TNF- α (1:500; R&D Systems, Minneapolis, MN, USA), TNFR1 (1:1000; Santa Cruz Biotechnology), TNFR2 (1:200; Santa Cruz Biotechnology), TH [1:5000; Chemicon (EMD Millipore), Temecula, MA, USA], β -actin (1:50000; Sigma-Aldrich), or COX IV (1:10000; Cell Signaling Technology, Danvers, MA, USA). The membranes were then incubated with HRP-conjugated secondary anti-rabbit IgG (1:1000, GE healthcare, Piscataway, NJ, USA), or anti-mouse IgG (1:1000,

Sigma-Aldrich) antibodies for 2 h. Subsequent visualization was performed using an enhanced chemiluminescence system (ECL Plus®, GE Healthcare, Arlington Heights, IL, USA). Relative band intensities were quantified with PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat, Marne la Vallée, France) and then normalized to that of β -actin (whole lysate or cytosolic fraction) or COX IV (mitochondrial fraction) (Shin et al., 2014).

2.8. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from striatal tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Reverse transcription reactions were carried out using RNA to cDNA EcoDry Premix (Clontech, Palo Alto, CA, USA) with a 1 h incubation at 42°C. PCR amplification was performed for 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 1 min. The primer sequences used for PCR amplification are listed in Table 1. PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light. RNA yields were quantified using PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat) (Ho et al., 2015).

2.9. Quantification of 4-hydroxynonenal (HNE)

The amount of lipid peroxidation was determined by measuring the level of 4-hydroxynonenal (HNE) using the OxiSelect™ HNE adduct ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions (Shin et al., 2014).

2.10. Quantification of protein carbonyl groups

The extent of protein oxidation was assessed by measuring the amount of protein carbonyl groups, which was determined spectrophotometrically according to the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure (Shin et al., 2014) as described by Oliver et al. (1987).

2.11. Quantification of reactive oxygen species (ROS) formation

ROS formation in the striatum was assessed by measuring the conversion from 2',7'-dichlorofluorescein diacetate (DCFH-DA) to dichlorofluorescein (DCF) (Nguyen et al., 2015). Briefly, the cytosolic or mitochondrial fraction was added to a tube containing 2 mL of PBS with 10 nmol of DCFH-DA dissolved in methanol. The reaction was incubated at 37°C for 3 h, after which its fluorescence was measured at 480 nm excitation and 525 nm emission wavelengths. DCF was used as a standard.

2.12. Mitochondrial membrane potential (MMP)

MMP was measured as previously described (Shin et al., 2014) using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1; Molecular Probes). This dye exists as a green fluorescent monomer at low membrane potential, but reversibly forms red fluorescent "J-aggregates" at polarized mitochondrial potentials.

2.13. Intramitochondrial Ca^{2+} level

Intramitochondrial Ca^{2+} level was measured as previously described (Shin et al., 2014). Briefly, mitochondrial fractions (250 μg) from striatal tissues were incubated in the presence of Rhod-2-AM (5 μM) for 60 min at 37°C and washed three times with Ca^{2+} -free Locke's solution. This reduced form of Rhod-2-AM is a colorless, nonfluorescent dye that has a net positive charge, which promotes sequestration in the mitochondria. After sequestration, the dye is oxidized, and the AM ester is cleaved, thereby trapping the dye in the mitochondria. The resultant fluorescence was quantified with a fluorescent plate reader (Molecular Devices), with excitation and emission wavelengths of 549 and 581 nm, respectively.

2.14. Complex I activity

Complex I activity was examined as described previously (Janssen et al., 2007). In short, isolated mitochondrial samples (μL) were added to reaction mixtures containing 25 mM potassium phosphate buffer (pH 7.8), 3.5 mg/mL bovine serum albumin, 60 μM 2,6-dichloroindophenol, 70 μM decylubiquinone, and 1 μM antimycin A. The reactions were then incubated at 37°C for 3 min. After adding NADH to a final concentration of 0.2 mM, the absorbance at 600 nm was recorded at 60 s intervals for 4 min. Next, rotenone was added to a final concentration of 1 μM , and the absorbance at 600 nm was again recorded at 60 s intervals for 4 min. One unit of complex I activity was defined as the reduction of 1 μmol of 2,6-dichloroindophenol per min. Activity was calculated based on an extinction coefficient for 2,6-dichloroindophenol of 19.1 $\text{mM}^{-1} \text{cm}^{-1}$. All activities are expressed as percentage of the control group activity.

2.15. Measurement of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and serotonin (5-HT)

Mice were sacrificed by cervical dislocation, and their brains were removed. The striata and prefrontal cortices were dissected, immediately frozen on dry ice, and stored at -70°C until the assays were performed. Tissues were weighed, ultrasonicated in 10% perchloric acid, and centrifuged at $20,000 \times g$ for 10 min. The levels of DA, DOPAC, HVA, and 5-HT were measured simultaneously using an HPLC instrument coupled with an electrochemical detector, as previously described (Jung et al., 2010; Shin et al., 2014).

2.16. Immunocytochemistry

Immunocytochemistry was performed as previously described (Shin et al., 2014). Mice were perfused transcardially with 50 mL of ice-cold PBS (10 mL/10 g body weight) followed by 4% paraformaldehyde (20 mL/10 g body weight). Brains were removed and stored overnight in 4% paraformaldehyde. A series consisting of every sixth section (35 μm thickness, 210 μm apart) of the striatum was selected and subjected to immunocytochemistry. Sections were blocked with PBS containing 0.3% hydrogen peroxide for 30 min and then incubated in PBS containing 0.4% Triton X-100 and 1% normal serum for 20 min. After 48 h of incubation with primary antibodies against MnSOD [1:1000; a kind gift from Dr. Kanefusa Kato, Aichi Prefectural Colony, Kasugai, Japan (Kim et al., 2000 and 2003; Kurobe and Kato, 1991)], TH [1:500; Chemicon (EMD Millipore)], or Iba-1 (1:500, Wako Pure Chemical Industries, Chuo-ku, Osaka, Japan), the sections were incubated with biotinylated

secondary antibodies (1:1000; Vector Laboratories, Burlingame, CA, USA) for 1 h. The sections were then immersed in a solution containing an avidin–biotin peroxidase complex (Vector Laboratories) for 1 h; 3,3'-diaminobenzidine was utilized as the chromogen. Digital images were acquired under an upright microscope (BX51; Olympus) using an attached digital microscope camera (DP72; Olympus) and an IBM-compatible PC.

ImageJ software, version 1.44 (National Institutes of Health, Bethesda, MD, USA) was employed to measure the immunoreactivities of MnSOD, TH, and Iba-1 in the striatum as described previously (Maximova et al., 2006; Pellegrino et al., 2007; Wang et al., 2012). Briefly, the entire striatal region from each section was selected as the region of interest (ROI). Hue, saturation, and brightness threshold values were set in the “Adjust Color Threshold” dialog box, and the mean density of each section was measured.

2.17. TUNEL staining

For TUNEL staining, a series consisting of every sixth section (35 μ m thickness, 210 μ m apart) of the striatum was selected. TUNEL staining was performed using a FragEL DNA fragmentation detection kit (QIA33; Calbiochem, La Jolla, CA, USA) according to the manufacturer's protocol and as described (Nguyen et al., 2015; Shin et al., 2012 and 2014; Zhang et al., 2006).

2.18. Statistics

Data were analyzed using IBM SPSS ver. 21.0 (IBM, Chicago, IL, USA). One-way analysis of variance (ANOVA) (PMMA time-point) or two-way ANOVA (PMMA $\times$ gene knockout or inhibitor) was employed to determine the significance of differences between groups. The P values are presented in Supplemental Tables 1–5. Post-hoc Fisher's least significant difference pairwise comparisons tests were then conducted. P-values < 0.05 were considered to be significant.

3. Results

3.1. PMMA selectively induces PKC δ expression over other PKC isoforms in the striatum of WT mice

Since protein kinase C (PKC) has been implicated in amphetamine-mediated behavioral and neurotoxic effects (Narita et al., 2008; Shin et al., 2012), we first investigated the expression of various PKC isoforms in the striatum of WT mice at 1 h, 12 h, 1 d, and 7 d after the final intraperitoneal injection (i.p.) of PMMA (60 mg/kg, twice a day for 4 consecutive days). PMMA selectively increased PKC δ expression at 12 h, 1 d, and 7 d after the final treatment (Fig. 1A and Supplemental Fig. S2). Thus, we focused on PKC δ for further study.

Repeated PMMA treatment significantly induced phospho-PKC δ expression. This expression peaked at 1 h and remained elevated 7 d later (Fig. 1A). In addition, PMMA significantly induced mitochondrial translocation of cleaved PKC δ at 1 h, 12 h, 1 d, and 7 d after treatment. This increase was most pronounced at 12 h post-PMMA treatment in WT mice (Fig. 1B). Although a single PMMA treatment also significantly induced phospho-PKC δ expression (Fig. 1C) and mitochondrial translocation of cleaved PKC δ (Fig. 1D),

these changes were less pronounced after a single PMMA treatment than after repeated PMMA treatments.

3.2. PKC δ knockout mice are protected from PMMA-induced mitochondrial burdens in the striatum

In our pilot study, we observed that PMMA induces oxidative burdens (Nam et al., 2015). To further investigate the kinetics of this effect, we performed time-course assessments of PMMA-induced oxidative damage. We measured cytosolic and mitochondrial oxidative stress in terms of protein oxidation (i.e., protein carbonyl groups), reactive oxygen species (ROS) level, and lipid peroxidation (i.e., HNE). Since PMMA-induced mitochondrial oxidative damage was most pronounced at 12 h after the final PMMA treatment in WT mice (Figs. 2A, B, and C), we focused on this time point for further study. In addition, PKC δ gene knockout significantly attenuated oxidative stress in the cytosol and mitochondria (Figs. 2A, B, and C).

Mitochondrial membrane potential (MMP), intramitochondrial Ca²⁺, mitochondrial complex I activity, mitochondrial MnSOD expression, and MnSOD immunoreactivity remained unchanged in the absence of PMMA. However, treatment with PMMA resulted in significantly decreased MMP, complex I activity, MnSOD expression, and MnSOD immunoreactivity in WT mice. In contrast, PMMA treatment resulted in significantly increased intramitochondrial Ca²⁺ level in WT mice. However, these changes were not significant in PKC δ KO mice, suggesting that PKC δ is critical for PMMA-mediated induction of mitochondrial dysfunction (Figs. 3A–E).

3.3. PKC δ knockout mice are protected from PMMA-induced transient dopaminergic loss in the striatum

Since PMMA and its metabolite, para-methoxyamphetamine (PMA), are closely related structurally, their mechanisms of action are presumably similar, primarily involving the release of serotonin from synaptic terminals (Steele et al., 1992). Treatment with PMMA resulted in a significant decrease in dopamine level, tyrosine hydroxylase-immunoreactivity (TH-IR), and TH expression at 12 h after treatment in WT mice. In contrast, PMMA treatment resulted in a significant increase in the dopamine turnover rate at the same time-point. This alteration was significantly attenuated in PKC δ KO mice (Figs. 4A–D). In contrast, PMMA did not significantly alter the dopamine level or turnover rate in the prefrontal cortex of WT or PKC δ KO mice (Supplemental Fig. 3). In addition, PMMA did not significantly alter serotonin level in the striatum or hippocampus of WT or PKC δ KO mice (Supplemental Figs. S4A and S4B). However, PMMA treatment did decrease the serotonin level in the prefrontal cortex of WT mice. This decrease was attenuated in PKC δ KO mice (Supplemental Fig. S4C).

3.4. PKC δ mediates PMMA-induced microglial activation in the striatum

Accumulating evidence suggests that mitochondrial damage links inflammation to neuronal death (Dang et al., 2016; Nguyen et al., 2015; Shin et al., 2014). Importantly, it has been proposed that microglia participate in neurotoxicity associated with methamphetamine intoxication (Dang et al., 2016; Shin et al., 2014; Wang et al., 2012 and 2014). In our study,

PMMA treatment increased Iba-1 expression in the striatum (12 h or 1 d post-PMMA, $p < 0.01$ vs. Sal). Moreover, PKC δ gene KO consistently and significantly attenuated PMMA-induced of Iba-1-labeled microglial cells (Fig. 5A and B). Therefore, genetic inhibition of PKC δ may dampen the neuroinflammatory process.

3.5. PMMA significantly and more selectively induces tumor necrosis factor alpha (TNF- α)-, and tumor necrosis factor receptor 1 (TNFR1) expression compared with interleukin 6 (IL-6)-, interferon gamma (INF- γ)-, and TNFR2- in the striatum of WT mice

Cytokines mediate inflammatory changes in the central nervous system (CNS) via specific receptor-mediated mechanisms (Rocha et al., 2015). Microglia produce numerous inflammatory cytokines including TNF- α , which plays a central role in initiating the proinflammatory response (Sadasivan et al., 2012). Therefore, we examined the mRNA and protein expression levels of various proinflammatory cytokines, such as IL-6, INF- γ and TNF- α at 1 h, 12 h, 1 d and 7 d after the final PMMA administration. We found that PMMA-induced changes in IL-6, INF- γ or TNF- α mRNA expression were comparable to the corresponding changes observed on the protein level (Figs. 6A–C). Treatment with PMMA resulted in significant increases in IL-6 (mRNA and protein; 1 h, 12 h, and 7 d; $P < 0.05$ vs. saline), INF- γ (mRNA and protein; 1 h, 12 h, 1 d, and 7 d; $P < 0.05$ vs. saline) and TNF- α (mRNA and protein; 1 h, 12 h, 1 d, and 7 d; $P < 0.01$ vs. saline) expression. Since TNF- α gene expression was most pronounced, we next examined which TNFR was specifically modulated by PMMA. PMMA treatment more significantly increased TNFR1 (mRNA and protein; 1 h, 12 h, 1 d, and 7 d; $P < 0.01$ vs. saline) expression to a greater extent than TNFR2 (mRNA and protein; 1 h, 12 h, and 1 d; $P < 0.05$ vs. saline) expression (Figs. 6D and E).

3.6. TNF- α knockout mice are protect PMMA-induced TH losses, phospho-PKC δ expression, mitochondrial translocation of cleaved PKC δ , and microglial activation in the striatum

To further elucidate the role of the proinflammatory cytokine TNF- α , we next studied the effects of PMMA treatment in TNF- α knockout mice. We found that TNF- α knockout mice were protected from PMMA-induced reductions in dopamine, TH-immunoreactivity and TH expression at 12 h post-PMMA ($P < 0.05$ vs. WT mice, respectively). Moreover, TNF- α knockout mice did not exhibit the elevated dopamine turnover rate at the same time-point (Figs. 7A–C). Importantly, TNF- α knockout significantly attenuated increases in phospho-PKC δ expression ($P < 0.05$ vs. WT mice) and mitochondrial translocation of cleaved PKC δ ($P < 0.05$ vs. WT mice) induced by PMMA. Consistently, TNF- α knockout significantly inhibited PMMA-induced Iba-1-labeled microglial activation (Iba-1-immunoreactivity and Iba-1 expression; $P < 0.05$ vs. WT mice, respectively) (Figs. 7D–F).

3.7. Pharmacological inhibitor of PKC δ (rottlerin) or TNF- α (etanercept) attenuates PMMA-mediated induction of TUNEL-positive cell staining in the striatum of Taconic ICR mice

Earlier reports have found that MA induces apoptotic cell death in striatal neurons (Deng and Cadet, 2000; Nguyen et al., 2015; Shin et al., 2012 and 2014). We performed TUNEL staining to detect DNA fragmentation, which occurs late in apoptosis. In accordance with

previous studies (Shin et al., 2012; Zhu et al., 2006a), we used 10-week-old male Taconic ICR mice. We sacrificed these mice at 12 h and 1 d after PMMA administration (80 mg/kg, i.p.), time-points that were chosen based on our pilot study (Nam et al., 2015). Representative photomicrographs of TUNEL-positive cells in the striatum of PMMA-treated mice are shown in Fig. 8. Very few TUNEL-positive cells were observed in the absence of PMMA. Moreover, treatment with rottlerin or etanercept significantly attenuated (12 h or 1 d post-PMMA, $p < 0.01$ vs. vehicle + PMMA) PMMA-mediated induction of TUNEL-positive cells, suggesting that the apoptotic effects of PMMA can be counteracted by pharmacological inhibition of PKC δ or TNF- α .

4. Discussion

We observed that, multiple doses of PMMA did not significantly alter PKC α , PKC β I, PKC β II, or PKC ζ expression in the striatum (Supplemental Fig. S2), but they did selectively and significantly increase PKC δ expression (Fig. 1A). PMMA initially stimulates phosphorylation and mitochondrial translocation of PKC δ . Consequently, PMMA promotes mitochondrial burdens, followed by microglial activation and initiation of pro-apoptotic signaling. This signaling process lasts for, at least, 1 day. We propose that this signaling process might be involved in the acute neurotoxic insult observed in the dopaminergic system at 12 h post-PMMA treatment.

Pharmacological or genetic inhibition of PKC δ protected against PMMA-induced neurotoxicity. Our results clearly suggest that PMMA-induced damage might be more pronounced in the mitochondria than in the cytosol of WT mice, and that genetic inhibition of PKC δ significantly attenuates oxidative damage, mitochondrial dysfunction, pro-apoptotic changes, and dopaminergic loss. However, it remains elusive whether PMMA directly binds PKC δ molecule for producing these neurotoxic events.

In rats, PMMA is metabolized in the liver by CYP2D6, a cytochrome P450 enzyme. It primarily undergoes *O*-demethylation to form para-hydroxymethamphetamine (Bach et al., 1999). PMA, and other metabolites are formed, albeit to a lesser extent for the latter (Rohanova and Balikova, 2009). Subcutaneous (s.c.) administration of PMMA (40 mg/kg) in rats results in peak serum PMMA concentration at 30 min, whereas the PMMA metabolite concentrations peak at 2 h. In the brain, the peak concentration can be reached 1 h after administration of PMMA, and 2 h after administration for PMA (Rohanova and Balikova, 2009). Therefore, we propose that a high level of PMMA or PMA in the brain may potentiate early mitochondrial oxidative stress (Fig. 2) and induce mitochondrial dysfunction (Fig. 3).

There are several additional ways in which sublethal mitochondrial disorders can cause subtle alterations in Ca²⁺ signaling that then affect neural function. These include increased ROS generation, impaired adenosine triphosphate (ATP) production, or altered mitochondrial Ca²⁺ buffering (Krieger and Duchen, 2002). Sustained increases in the basal rate of mitochondrial ROS formation have been shown to lead to a gradual and progressive depletion of cellular antioxidant defenses, perhaps increased mitochondrial Ca²⁺, thereby further promoting ROS generation (Dykens, 1994; Krieger and Duchen, 2002).

Increased intracellular Ca^{2+} promotes the accumulation of Ca^{2+} within the mitochondrial matrix when the total Ca^{2+} uptake exceeds total Ca^{2+} efflux from mitochondria (Nicholls, 2009). Mitochondrial Ca^{2+} overload can lead to the uncoupling of mitochondrial electron transport and potentiate oxidative stress. The decreases in MMP and increase in oxidative damage after PMMA treatment could have been mediated by Ca^{2+} entry, suggesting that inhibition of PKC δ blocks Ca^{2+} entry.

Cassarino et al. (1999) demonstrated that the mechanisms by which mitochondrial complex I impairment leads to apoptotic signaling include production of superoxide radical anions, mitochondrial swelling, loss of energy supply, and oxidative burdens via mitochondrial transition pore opening (Cassarino et al., 1999; Testa et al., 2005). Thus, PMMA-induced decreases of MMP, complex I activity, and mitochondrial SOD-immunoreactivity and mitochondrial SOD expression, in addition increased mitochondrial oxidative damage, appear to be the first steps in pro-apoptotic signaling. Therefore, mitochondria are key for PMMA-induced initiation of oxidative damage, PKC δ activation, and apoptosis.

Mitochondrial toxins (e.g., 3-nitropropionic acid) induce microglial activation and oxidative stress, leading to neuronal loss in the striatum (Ryu et al., 2003). Activated microglia release free radicals that target mitochondria, leading to further mitochondrial destruction. This combination of mitochondrial dysfunction, oxidative stress and excessive microglial activation generates a vicious cycle that appears to lead to progressive neuronal cell death (Hald and Lotharius, 2005). More importantly, caspase activation regulates microglia activation through a PKC δ -dependent pathway (Burguillos et al., 2011).

Since microglia secrete cytotoxic agents, such as free radicals, cytokines and chemokines, it is generally accepted that microglial activation induces neurotoxicity. Although PMMA treatment did not produce long-lasting dopaminergic loss, we observed significant induction of microgliosis and increased TUNEL-positive cell populations. Furthermore, the early PMMA-induced oxidative stress observed in WT mice returned to near control levels after 7 days. These phenomena could be attributable to the positive effects of unknown neurotropic/antioxidant/anti-inflammatory substances (Miyazaki et al., 2011; Zhang et al., 2006). In addition, we propose that PKC δ inhibition significantly facilitates antioxidant and neurotropic potential by positively modulating mitochondrial functions and other unknown mechanisms.

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 , which appears to play a central role in initiating the proinflammatory response (Sadasivan et al., 2012). Here we observed that TNF- α plays a major role in the neuroinflammatory effects, proapoptotic changes and dopaminergic loss mediated by PKC δ , although PMMA also upregulated IL-6 and INF- γ expression. We suggest that proapoptotic PKC δ -induced neurotoxicity requires the activation of intracellular TNF- α , which may be consistent with previous studies (McCoy et al., 2006; McGuire et al., 2006) concluding that TNF- α can induce dopaminergic neurotoxicity through a direct mechanism, possibly via the TNFR1 death receptor.

Since microglial activation is known to prolong immune stimulation, thereby leading to the progressive neuroinflammation implicated in neurodegenerative disorders (Block and Hong, 2005; Block et al., 2007; Gao and Hong, 2008), microglial activation via PKC δ or TNF- α (Figs 5 and 7) might contribute to PMMA-induced neurotoxicity. Thus, we propose that activation of PKC δ or TNF- α is conducive to maintaining a subpopulation of microglia in their proinflammatory phenotype, thereby exerting detrimental effects on dopaminergic neurons in PMMA-treated brain.

We found that PMMA treatment produced serotonergic loss in prefrontal cortex, but not in the striatum or hippocampus (Supplemental Fig. S4). These findings are consistent with those from earlier study in rats (Steele et al., 1992). It is well-documented that MA produces long-term damage to dopaminergic and serotonergic axon terminals in the striatum, hippocampus, and prefrontal cortex (Ricaurte et al., 1980; Wagner et al., 1980; Seiden et al., 1988). Therefore, we conclude that PMMA is a less potent dopaminergic neurotoxin than methamphetamine (MA). It might be required *in vivo* microdialysis for detecting precise change in dopamine level for our future study. However, we observed that PMMA-induced dopaminergic impairments require PKC δ activation as was the case for MA, and that TNF- α mediates PMMA-induced dopaminergic neurotoxicity. However, our earlier study clearly demonstrated that TNF- α plays a neuroprotective role in response to MA-induced dopaminergic neurotoxicity (Nakajima et al., 2004). The explanation behind this discrepancy remains to be fully elucidated.

Taken together, our data indicate that PKC δ mediates PMMA-induced neurotoxicity by facilitating oxidative burdens (mitochondria > cytosol), mitochondrial dysfunction, microglial activation, pro-apoptotic signaling, and dopaminergic impairment. Moreover, our finding suggest that TNF- α activates PMMA-induced PKC δ signaling, and that interactive modulation between PKC δ and TNF- α is critical for PMMA neurotoxicity. Since PKC δ activation might be a common target for multiple mediators of dopaminergic degeneration in neuroinflammation, PKC δ is an attractive therapeutic target for the treatment of dopaminergic neurodegenerative disorders such as Parkinson's disease and amphetamines' neurotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
ATP	adenosine triphosphate

CNS	central nervous system
DA	dopamine
DCF	dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein diacetate
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'- tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNE	4- hydroxynonenal
HVA	homovanillic acid
Iba-1	ionized calcium binding adaptor molecule 1
IFN-γ	interferon gamma
IL-6	interleukin 6
KO	knockout
MA	methamphetamine
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxyethylamphetamine
MMP	mitochondrial membrane potential
MnSOD	manganese-dependent superoxide dismutase
PKCδ	protein kinase C δ
PMA	para-methoxyamphetamine
PMMA	paramethoxymethamphetamine
ROS	reactive oxygen species
PCR	polymerase chain reaction
TH	tyrosine hydroxylase
TH-IR	TH-immunoreactivity
TNF-α	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor

TUNEL	terminal deoxynucleotidyl transferase dUDP nick end labeling
WT	wild-type

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Highlights

- PMMA facilitates phosphorylation and mitochondrial translocation of PKC δ .
- PMMA potentiates oxidative stress (mitochondria > cytosol).
- PKC δ knockout protects “oxidative stress and mitochondrial dysfunction” by PMMA.
- PKC δ knockout protects “dopaminergic impairment, microgliosis and apoptosis” by PMMA.
- PMMA-induced PKC δ signaling requires activation of TNF- α gene.
- Interactive modulation between PKC δ and TNF α is critical for PMMA neurotoxicity.

Fig. 1.

Time-dependent alterations in phosphorylation of PKCδ (A and C) and mitochondrial translocation of cleaved PKCδ (B and D) in the striata of wild type (WT) mice induced by multiple doses (A and B) or a single dose (C and D) of PMMA. Sal = Saline. Each value is the mean $\pm$ S.E.M. of 6 animals. One-way ANOVA showed a significant effect of PMMA time-point on phosphorylation of PKCδ (A and C) and mitochondrial translocation of cleaved PKCδ (B and D) activation (Supplemental Table 1). * $p < 0.05$, ** $p < 0.01$ vs. Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 2.

PMMA-induced time-dependent alterations in cytosolic and mitochondrial protein oxidation (A), lipid peroxidation (B), and reactive oxygen species (ROS; C) in the striata of wild type and PKC δ KO mice. Sal = Saline. WT = wild type. Each value is the mean $\pm$ S.E.M. of 6 animals. Two-way ANOVA showed significant effects of PMMA time-point (A-C) and PKC δ KO (A-C). In addition, two-way ANOVA revealed a significant interaction between PMMA time-point and PKC δ KO (mitochondrial protein oxidation and mitochondrial lipid peroxidation) (Supplemental Table 1). * p < 0.05, ** p < 0.01 vs. WT with saline. # p < 0.05, ## p < 0.01 vs. the corresponding time-point of WT with PMMA (two-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 3.

PMMA-induced alterations in mitochondrial membrane potential (A), intramitochondrial Ca²⁺ level (B), mitochondrial complex I activity (C), mitochondrial Mn-superoxide dismutase (MnSOD) expression (D), and MnSOD immunoreactivity (E) in the striata of wild type (WT) and PKCδ KO mice. Sal = Saline. WT = wild type. Each value is the mean ± S.E.M. of 6 animals. Two-way ANOVA showed significant effects of PMMA (A-E) and PKCδ KO (A-E). Two-way ANOVA also showed a significant interaction between PMMA and PKCδ KO (B) (Supplemental Table 2). *p < 0.05, **p < 0.01 vs. WT with saline. #*p* < 0.05 vs. WT with PMMA (two-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 0.5 mm.

Fig. 4.

PMMA-induced alterations in dopamine (A), dopamine turnover rate (B), tyrosine hydroxylase (TH) expression (C), and TH-immunoreactivity (D) in the striata of wild type and PKCδ KO mice. Sal = Saline. WT = wild type. Each value is the mean $\pm$ S.E.M. of 6 animals. Two-way ANOVA showed significant effects of PMMA time points (C) or PMMA (D) and PKCδ KO (B-D), and a significant interaction between PMMA time points and PKCδ KO (B). Additional statistical analysis with two-way ANOVA revealed significant effects of PMMA (A and B) and PKCδ KO (A and B), and a significant interaction between PMMA and PKCδ KO (A and B) at 12 h post-PMMA (Supplemental Table 2). * $p < 0.05$ vs. WT with saline. # $p < 0.05$ vs. the corresponding time-point of WT with PMMA (two-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 1 mm.

Fig. 5.

PMMA-induced alterations in Iba-1-expression (A) and Iba-1 immunoreactivity (B) in the striata of wild type and PKCδ KO mice. Sal = Saline. WT = wild type. Each value is the mean ± S.E.M. of 6 animals. One-way ANOVA showed a significant effect of PMMA time-point on the Iba-1 expression (A). In addition, two-way ANOVA showed significant effects of PMMA (B) and PKCδ KO (B), and a significant interaction between PMMA and PKCδ KO (B) (Supplemental Table 3). * $p < 0.01$ vs. Sal or WT with saline. # $p < 0.01$ vs. WT with PMMA [one-way ANOVA (A) or two-way ANOVA (B) followed by Fisher's LSD pairwise comparisons]. Scale bar = 100 μm.

Fig. 6.

PMMA-induced alterations in mRNA and protein levels of IL-6 (A), INF- γ (B), TNF- α (C), TNFR1 (D) and TNFR2 (E) in the striata of wild type mice. Each value is the mean $\pm$ S.E.M. of 6 animals. One-way ANOVA showed a significant effect of PMMA time-point on the mRNA and protein levels of IL-6 (A), INF- γ (B), TNF- α (C), TNFR1 (D) and TNFR2 (E) in the striata of wild type mice (Supplemental Table 3). * $p < 0.05$, ** $p < 0.01$ vs. Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 7.

PMMA-induced alteration in dopamine level and TH expression (A), dopamine turnover rate (B), TH-immunoreactivity (C), phospho-PKCδ expression (D), mitochondrial translocation of cleaved PKCδ (E), and Iba-1-immunoreactivity and expression (F) in the striata of wild type (WT) and TNF-α KO mice. Each value is the mean ± S.E.M. of 6 animals. Two-way ANOVA showed significant effects of PMMA (A-F) and PKCδ KO (A-F), and a significant interaction between PMMA and PKCδ KO (B and D-F) (Supplemental Table 4). * $p < 0.05$, ** $p < 0.01$ vs. WT with saline. # $p < 0.01$ vs. WT with PMMA (two-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 1 mm (C) or 100 μm (F).

TUNEL-positive cells (12h and 1d post-PMMA)

Fig. 8.

Effect of rottlerin (Rot), or etanercept (Eta) on PMMA-induced apoptotic cell death. Apoptotic cell death was observed as an increase in TUNEL-positive cells in the striata of Taconic ICR mice. Sal = Saline. Veh = vehicle [10% (v/v) DMSO] for rottlerin. Rot = rottlerin (3μg, i.c.v.). Eta = etanercept (50μg, i.c.v.). Each value is the mean ± S.E.M. of 6 animals. Two-way ANOVA showed significant effects of PMMA and pretreatment with rottlerin or etanercept, and a significant interaction between PMMA and pretreatment (Supplemental Table 4). * $p < 0.01$ vs. vehicle + saline. # $p < 0.01$ vs. vehicle + PMMA (two-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 100 μm.

Table 1.

Gene primer sequences and PCR conditions for RT-PCR analysis.

Gene	Primer sequences (5'- 3')	PCR condition (denature/annealing/extension)	Cycles	Expected size (bp)
TNF- α	F: AGCACAGAAAGCATGATCCG R: ACTCCAGCTGCTCCTCCACT	94°C, 30 sec/ 58°C, 30 sec/ 72°C, 1 min	32	316 bp
IL-6	F: TGGAGTCACAGAAGGAGTGGCTAAG R: TCTGACCACAGTGAGGAATGTCCAG	94°C, 30 sec/ 59°C, 30 sec/ 72°C, 1 min	35	155 bp
INF- γ	F: AGGAACTGGCAAAAGGATGGTG R: CTGGACCTGTGGGTTGTTGA	94°C, 30 sec/ 58°C, 30 sec/ 72°C, 30 sec	33	219 bp
TNFR1	F: CCGGGCCACCTGGTCCG R: CAAGTAGGTTCTTTGTG	94°C, 1 min 30 sec/ 55°C, 2 min/ 72°C, 2 min	35	307 bp
TNFR2	F: CTCGCGCTGGTCTTCGAACTG R: GGTATACATGCTTGCCCTCACAGTC	94°C, 1 min/ 62°C, 1 min/ 72°C, 2 min	35	234 bp