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PKC δ -dependent p47 $phox$ activation mediates methamphetamine-induced dopaminergic neurotoxicity

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Abstract

Protein kinase C (PKC) has been recognized to activate NADPH oxidase (PHOX). However, the interaction between PKC and PHOX *in vivo* remains elusive. Treatment with methamphetamine (MA) resulted in a selective increase in PKC δ expression out of PKC isoforms. PKC δ co-immunoprecipitated with p47 $phox$, and facilitated phosphorylation and membrane translocation of p47 $phox$. MA-induced increases in PHOX activity and reactive oxygen species were attenuated by knockout of p47 $phox$ or PKC δ . In addition, MA-induced impairments in the Nrf-2-related glutathione synthetic system were also mitigated by knockout of p47 $phox$ or PKC δ . Glutathione-immunoreactivity was co-localized in Iba-1-labeled microglial cells and in NeuN-labeled neurons,

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but not in GFAP-labeled astrocytes, reflecting the necessity for self-protection against oxidative stress by mainly microglia. Buthionine-sulfoximine, an inhibitor of glutathione biosynthesis, potentiated microglial activation and pro-apoptotic changes, leading to dopaminergic losses. These neurotoxic processes were attenuated by rottlerin, a pharmacological inhibitor of PKC δ , genetic inhibitions of PKC δ [i.e., PKC δ knockout mice (KO) and PKC δ antisense oligonucleotide (ASO)], or genetic inhibition of p47 $phox$ (i.e., p47 $phox$ KO or p47 $phox$ ASO). Rottlerin did not exhibit any additive effects against the protective activity offered by genetic inhibition of p47 $phox$. Therefore, we suggest that PKC δ is a critical regulator for p47 $phox$ activation induced by MA, and that Nrf-2-dependent GSH induction via inhibition of PKC δ or p47 $phox$, is important for dopaminergic protection against MA insult.

Graphical Abstract

Keywords

Methamphetamine; Dopamine; Striatum; PKC δ knockout mice; PKC δ antisense oligonucleotide; p47 $phox$ knockout mice; p47 $phox$ antisense oligonucleotide; Nrf2-transcription factor; Glutathione-immunoreactivity; Microglia; Pro-apoptosis

1. Introduction

NADPH oxidase (PHOX) is a multi-unit enzyme complex responsible for the production of both extracellular and intracellular reactive oxygen species (ROS) by phagocytic cells, including microglia. PHOX is comprised of cytoplasmic subunits (p47 $phox$, p67 $phox$, p40 $phox$, and Rac2), which, upon phosphorylation by specific kinases, can form a complex and translocate to the membrane to dock with the membrane subunits (gp91 $phox$, and

p22 $phox$) [1]. It is well-recognized that p47 $phox$ acts as a connector between the components of the membrane and the cytoplasm [2]. Therefore, key events of the activation of PHOX are the phosphorylation and translocation of p47 $phox$.

It is recognized that a number of kinases have been implicated in the activation of the oxidase, including protein kinase C (PKC) [2]. PKC is a family of multiple isozymes that play a crucial role in the regulation of cellular proliferation and differentiation [3]. The PKC family is divided into several subfamilies, and individual members have a tissue-specific distribution and mode of activation, as well as kinetics and substrate specificities, suggesting that individual or subgroups of PKC isozymes play discrete roles within cells [4]. However, different and conflicting effects of PKCs on apoptosis in various model systems have made it difficult to define their role in toxicity [5].

It has been well-recognized that high doses of methamphetamine (MA) result in impaired dopaminergic systems in rodents [6–10] and primates [11]. Although the pathogenesis of the MA-induced dopaminergic neurotoxicity remains to be further elucidated, this neurotoxicity may be, at least in part, related to oxidative stress [9,10,12–16], inflammatory changes [10,12,17,18], and pro-apoptosis [10,15,16,19–22].

Furthermore, previous investigations have indicated that humans who abuse MA have an increased risk for PD later in life [23–26]. Earlier postmortem studies reported reductions in dopamine (DA) level, tyrosine hydroxylase (TH) expression, and dopamine transporter (DAT) binding in the striatum of the MA abuser [27], and these changes paralleled neurochemical changes in PD patients [28,29].

We have reported that multiple doses of MA do not significantly alter PKC α , PKC β I, PKC β II, or PKC ζ expression in the striatum, but do significantly and selectively increase PKC δ expression [15]. We have demonstrated that MA-induced enhancement of PKC δ expression is a critical factor in impaired TH phosphorylation [15,30]. We have also suggested that PKC δ mediates MA-induced dopaminergic neurodegeneration by potentiating oxidative damage and inhibiting TH phosphorylation [15,30], and that pharmacological or genetic inhibition of PKC δ is protective against MA-induced impairment in the glutathione-dependent system *in vivo* [10,30]. Similarly, we have reported that pharmacological or genetic inhibition of PKC δ attenuates neuroexcitotoxicity through an Nrf-2-dependent glutathione defense mechanism [31].

Microglia-mediated neuroinflammation has been linked to multiple neurodegenerative diseases, including PD [32–39]. One recent therapeutic strategy has been to deviate from conventional anti-inflammatory targets and inhibit upstream mediators, such as PHOX [33]. Once activated, PHOX produces extracellular and intracellular reactive oxygen species, which are critical in initiating and maintaining neuroinflammatory responses, leading to progressive dopaminergic neurodegeneration [35,36,40]. For example, activated microglia secrete a variety of toxic factors, such as tumor necrosis factor α , interleukin-1 and other pro-inflammatory cytokines, which work in concert to cause neuronal damage [34]. Hong and colleagues have recognized PHOX as a key mediator in bridging neuroinflammation and progressive dopaminergic neurodegeneration [35,36,41,42].

Park et al. [43] demonstrated that MA (10 μ M) induces an increase in phosphorylation of the p47 $phox$ subunit, and subsequently enhances PHOX activity in endothelial cells. Recently, we demonstrated that PHOX inhibitor apocynin protects mitochondrial burdens, microglial activation, and proapoptotic signaling via ERK-dependent activation of p47 $phox$ [44]. However, the role of PKC δ in the activation of PHOX remains unknown *in vivo*. The purpose of the current study is to determine in advance what kinds of PKC isoforms are involved in regulating PHOX activity against acute neurotoxicity induced by MA. We observed that PKC δ is selectively involved in regulating activity of p47 $phox$. Here, we propose that the interactive signaling process between PKC δ and p47 $phox$ contributes to oxidative burdens, impairment in Nrf-2-related glutathione induction, microglial activation, pro-apoptosis, and dopaminergic loss induced by MA.

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, and the animal experimental procedure was approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (#KIACUC-12-0016). 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.

Wild-type C57BL/6 and p47 $phox$ knockout mice (KO) were purchased from Jackson Laboratories (Bar Harbor, Maine, U.S.A.) [44,45].

A breeding pair of PKC δ (+/–) mice, originally bred into a C57BL/6 background, was a gift from Dr. K.I. Nakayama (Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan) [46]. 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 (+/+) mice from the same litter in our experiments. The details of the genotyping procedure are provided in the supplementary information section.

To evaluate the effect of buthionine-sulfoximine on the pharmacological activity of rottlerin, PKC δ antisense oligonucleotide, or p47 $phox$ antisense oligonucleotide on the MA-induced pro-apoptosis, we employed 10-week-old male ICR mice (Taconic Farms, Inc., Samtako Bio Korea, O-San, South Korea), because our previous reports [10,14,15] indicated that the C57BL/6 background does not exhibit MA-induced terminal deoxynucleotidyl transferase dUDP nick end labeling (TUNEL)-positive cells in the striatum, but does so in Taconic ICR mice.

2.2. Drug treatment

Although the 4 $\times$ 7–10 mg/kg paradigm of MA administration is currently the most frequently used model that mimics an acute toxic dose of MA [12], we selected a toxic dose (35 mg/kg, i.p.) of MA as we described elsewhere [44,47,48] because we demonstrated that

this paradigm is more sensitive than the 4×7 mg/kg paradigm for producing more significant dopaminergic protective effects by apocynin or p47 $phox$ gene knockout [44].

Mice were treated with a single dose of MA (35 mg/kg, i.p.) or saline, and sacrificed 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 1 d, 3 d, and 7 d after MA treatment to examine phosphorylation and membrane translocation of p47, Nrf-2 nuclear translocation and DNA binding activity, PHOX activity, ROS formation, cleavage and phosphorylation of PKC δ , co-immunoprecipitation of PKC δ and p47 $phox$, glutathione (GSH) and glutathione disulfide (GSSG) levels, mRNA expression of γ -glutamylcysteine ligase catalytic subunit (GCLc) and γ -glutamylcysteine ligase modifier subunit (GCLm), changes in TH-, ionized calcium binding adaptor molecule 1 (Iba-1)-, PKC α -, PKC β I-, PKC β II-, PKC ζ -, and PKC δ -expression, and dopamine and dopamine turnover rate. Rottlerin (PKC δ inhibitor; Biomol Research Laboratories, Inc., Plymouth, PA, U.S.A.) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and then stored at -20°C . Rottlerin was diluted in sterile saline immediately prior to use at a concentration of $1 \mu\text{g}/\mu\text{L}$. The final DMSO concentration was 10% (v/v). Rottlerin ($3 \mu\text{g}$, i.c.v.) was given 6 and 2 h before MA injection. The dose of rottlerin was determined based on previous studies [10,15]. Buthionine-sulfoximine (BSO) (inhibitor of glutathione biosynthesis; Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in sterile saline. BSO ($3 \mu\text{mol}/3 \mu\text{L}$, i.c.v.) was given 21 h after MA injection. The dose of BSO was determined based on previous study [49].

2.3. Guide cannula building and intracerebroventricular infusion with p47 $phox$ sense oligonucleotide (p47 $phox$ SO) or p47 $phox$ antisense oligonucleotide (p47 $phox$ ASO), and PKC δ sense oligonucleotide (PKC δ SO) or PKC δ antisense oligonucleotide (PKC δ ASO)

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 to the midline, and 2 mm ventral to the dura, according to the atlas of Franklin and Paxinos) as described previously [10,14]. One day after guide cannula implantation, Taconic ICR mice received a single injection of MA (35 mg/kg, i.p.). p47 $phox$ ASO (5'-GGTGTCCCCCATGGCTGGGCCG) or control p47 $phox$ SO (5'-CGGCCAGCCATGGGGGACACC) [GenBank accession number: [AB002663.1](#)] was microinfused into the lateral ventricle at a dose of $2.5 \mu\text{g}/\mu\text{L}$ at 4 and 0.5 h before, and at 4 h after MA injection. PKC δ ASO (5'-GAAGGCGATGCGCAGGAA) or control PKC δ SO (5'-AGGAACGGCGCCATGGTGGG) was microinfused into the lateral ventricle at a dose of $2.5 \mu\text{g}/\mu\text{L}$ at 4 and 0.5 h before, and at 4 h after MA injection. P47 $phox$ SO, p47 $phox$ ASO, PKC δ SO, and PKC δ ASO used here were phosphorothioated on the two terminal bases of the 5'-end and three terminal bases of the 3'-end (Bioneer Corporation, Daejeon, South Korea). 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.

2.4. Brain dissection

Mice were sacrificed by decapitation, and brains were rapidly removed and placed on an ice-cold brain matrix (ASI Instruments, Warren, MI, U.S.A.). Coronal slices containing striatum

were made at 1.1 to -0.1 mm from bregma using chilled razor blades, according to the atlas of Franklin and Paxinos [50]. Dorsal striatum was punched bilaterally with a sample corer (2 mm inner diameter for striatum; Fine Science Tools Inc., Vancouver, Canada) and a plunger [51,52]. Dissected tissues were frozen in liquid nitrogen and stored at -80°C until use.

2.5. Preparation of cytosolic and membrane fractions for Western blot analysis

Cytosolic and membrane fractions were prepared as described previously with minor modifications [53]. Animal tissues were collected and homogenized in ice-cold lysis buffer (pH 7.4) containing 25 mmol/L Tris, 250 mmol/L NaCl, 3 mmol/L ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (Sigma-Aldrich) using a Dounce homogenizer. The lysates were loaded onto sucrose in the lysis buffer and centrifuged at $1,600 \times g$ for 15 min; the supernatant above the sucrose gradient was utilized as the cytosolic fraction after centrifugation at $150,000 \times g$ for 30 min at 4°C . The resulting pellets were resuspended with lysis buffer containing 1% Triton X-100 and used as the membrane fraction.

2.6. Nuclear extraction for the evaluation of nuclear translocation of Nrf-2

Nuclear and cytosolic fractions of striatal lysates were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, IL, U.S.A.) according to the manufacturer's instructions. Briefly, tissues were homogenized in the provided cytoplasmic extraction reagent using a Dounce homogenizer. The homogenate was centrifuged at $16,000 \times g$ for 5 min, and the supernatant (cytosolic) fraction was immediately transferred to a pre-chilled tube. The pelleted fraction was suspended in the provided nuclear extraction reagent (pre-chilled), and the resulting suspension was centrifuged at $16,000 \times g$ for 10 min. The supernatant (nuclear) fraction was immediately transferred to a pre-chilled tube. The cytosolic and nuclear fractions were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ($20 \mu\text{g}$ protein/lane), and the separated proteins were transferred onto the polyvinylidene fluoride (PVDF) membrane.

2.7. Western blot analysis

Striatal tissues were lysed in buffer containing a 200 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM EDTA, 10% glycerol, 1 $\times$ phosphatase inhibitor cocktail I (Sigma-Aldrich, St. Louis, MO, U.S.A.), and 1 $\times$ protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, U.S.A.). Lysate was centrifuged at $12,000 \times g$ for 30 min, and the supernatant fraction was utilized for Western blot analysis as described previously [10,15]. Proteins ($20 \mu\text{g}$ /lane) were separated by 8% or 10% SDS-PAGE, and transferred onto PVDF membranes. Following transfer, the membranes were preincubated with 5% non-fat milk for 30 min and incubated overnight at 4°C with primary antibody against p47^{phox} [1:500; Chemicon (EMD Millipore), Temecula, MA, U.S.A.], p-p47^{phox} at Ser345 (1:1000; Sigma-Aldrich, St. Louis, MO, U.S.A.), Na⁺/K⁺-ATPase $\alpha 1$ subunit (1:1000; Abcam, Cambridge, UK), PKC α (1:70000; Santa Cruz Biotechnology), 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), p-PKC δ at Tyr311 (1:500; Santa Cruz Biotechnology), cleaved PKC δ (1:2000; Santa Cruz Biotechnology), Nrf-2 (1:1000; Santa Cruz Biotechnology), nuclear

marker (1:1000; Abcam, Cambridge, UK), p-Akt at Ser473 (1:2000; Cell Signaling Technology), p-Bad at Ser136 (1:1000; Cell Signaling Technology), 14-3-3 (1:1000; Santa Cruz Biotechnology), Bcl-2 (1:1000; Santa Cruz Biotechnology), Bcl-xL (1:1000; Cell Signaling Technology), Bax (1:1000; Santa Cruz Biotechnology), cleaved caspase 3 (1:1000; Cell Signaling Technology), Iba-1 (1:500; Abcam, Cambridge, UK), NeuN (1:200; EMD Millipore), GFAP (1:500; Santa Cruz Biotechnology), TH [1:5000; Chemicon (EMD Millipore)], or β -actin (1:50000; Sigma-Aldrich, St. Louis, MO, U.S.A.). Membranes were then incubated with HRP-conjugated secondary anti-rabbit IgG (1:1000, GE Healthcare, Piscataway, NJ, U.S.A.), anti-mouse IgG (1:1000, Sigma-Aldrich), or anti-goat IgG (1:1000, Sigma-Aldrich) for 2 h. Subsequent visualization was conducted using an enhanced chemiluminescence system (ECL plus®, GE Healthcare, Arlington Heights, IL, U.S.A.). Relative intensities of the bands were quantified by PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat, Marne la Vallée, France), and then normalized to the intensity of β -actin (whole lysate or cytosolic fraction), nuclear marker (nuclear fraction), or Na^+/K^+ -ATPase $\alpha 1$ subunit (membrane fraction) [10].

2.8. Nrf-2 DNA-binding activity

The nuclear fraction was extracted using a nuclear extraction kit (#40410; Active Motif, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Briefly, fresh striatal tissue was homogenized in the hypotonic buffer provided in the kit, and the homogenate was incubated on ice for 15 min. After centrifugation for 10 min at $850 \times g$, the pellet was resuspended in the complete lysis buffer provided in the kit. This suspension was incubated for 30 min on ice and then centrifuged for 10 min at $14,000 \times g$ at 4°C . The supernatant (nuclear fraction) was stored at -80°C until use. The protein concentration was determined using the BCA protein assay kit (Thermo Scientific).

Nrf-2 DNA-binding activity was measured using the TransAM® Nrf2 transcription factor ELISA kit (Active Motif) [54] according to the manufacturer's instructions. Briefly, $10 \mu\text{g}$ of each nuclear protein extract were added to wells coated with oligonucleotides containing an antioxidant responsive element (ARE) consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3'). The plate was incubated for 1 h at room temperature and then washed with the $1\times$ wash buffer provided in the kit. After incubation with the primary antibody against Nrf-2 for 1 h at room temperature, the plate was incubated with a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG for 1 h. The colorimetric reaction was initiated using the developing solution provided in the kit. The absorbance at 450 nm was measured using a microplate reader (Spectra Max Plus 384, Molecular Devices, Sunnyvale, CA, U.S.A.).

2.9. Immunoprecipitation studies

Immunoprecipitation was performed as described previously [55] using protein G-sepharose (GE Healthcare, Piscataway, NJ, U.S.A.). Striatal tissues were homogenized in lysis buffer for 1 min on ice and centrifuged at $12,000 \times g$ for 20 min at 4°C to remove particulate matter. The supernatant fractions were transferred to new tubes in 1-ml aliquots and pre-cleared by the addition of $50 \mu\text{l}$ of protein G-sepharose suspension (GE Healthcare) to each tube, gentle mixing for 1 h at 4°C , and centrifugation at $12,000 \times g$ for 20 s. Five hundred

microliter portions of the pre-cleared supernatant fractions were then removed to new tubes and incubated with 5 µg of an antibody specific for p-Bad at Ser136 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) or PKCδ (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A) for 1 h at 4°C to allow the formation of immune complexes. To precipitate the immune complexes, 50 µl of protein G-sepharose suspension was added to each tube, and the tubes were incubated for 5 h at 4°C. The immune complexes bound to sepharose beads were recovered by centrifugation and washed three times with wash buffer to remove the excess cytosolic fraction.

For analysis, the precipitated beads were mixed with SDS-PAGE sample buffer and heated for 10 min at 95°C. The dissociated proteins were then resolved by 10% SDS-PAGE and detected by immunoblotting with an antibody specific for 14-3-3 (1:1,000; Santa Cruz Biotechnology) or p47^{phox} [1:500; Chemicon (EMD Millipore), Temecula, MA, U.S.A.].

2.10. NADPH oxidase (PHOX) activity

Lucigenin-enhanced chemiluminescence method was used in measuring the enzyme activity of PHOX in tissue homogenates, as described previously with minor modifications [56,57]. Briefly, animal tissues were homogenized in a detergent-free lysis buffer (pH 7.4) containing 150 mmol/L NaCl, 50 mmol/L Tris, 25 mmol/L EGTA, 25 mmol/L EDTA and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, U.S.A.) using a Dounce homogenizer. A total of 2 mg/mL of the protein homogenate, diluted in the same Tris buffer, was utilized for each experiment. Immediately before the experiment, NADPH (200 µM) and lucigenin (10 µM) were added from stock solutions. Each sample was incubated at 37°C, and the lucigenin-dependent light emission was detected by a photomultiplier-based luminometer. For each sample, 30 s integrated luminescence was measured and repeated for five times. The activity of PHOX was calculated as the average of the five repeats, and was expressed as relative luminescence units per minutes per mg protein.

2.11. Synaptosome preparation

Striatal tissue was homogenized in 10 volume of ice-cold 0.32 mol/L sucrose and centrifuged at low speed ($800 \times g$, 12 min, 4°C). This resulted in supernatant (S1) that was removed and centrifuged at high speed ($22,000 \times g$, 20 min, 4°C) in order to yield pelleted synaptosomes. An aliquot of the re-suspended (P2) synaptosomal fraction was used for determination of ROS formation [15].

2.12. Determination of ROS formation

The ROS formation in the synaptosome was assessed by measuring the conversion from 2',7'-dichlorofluorescein diacetate (DCFH-DA) to dichlorofluorescein (DCF) [14]. Synaptosomal fractions were added to a tube containing 2 mL of phosphate-buffered saline (PBS) with 10 nmol of DCFH-DA, dissolved in methanol. The mixture was incubated at 37°C for 3 h, and then fluorescence was measured at 480 nm excitation and 525 nm emission. DCF was used as a standard.

2.13. Determination of GSH and GSSG by HPLC

Striatum were dissected immediately after decapitation, and GSH and GSSG were immediately measured from dissected tissues by the method described by Reed et al. [58] with minor modification [59,60]. Briefly, a sample of the acidified supernatant was added to the internal standard (1 mM cysteic acid) and 0.88 M iodoacetic acid. Excess potassium hydrogen bicarbonate was added to the reaction to precipitate potassium perchlorate. Subsequently, 0.5 ml of an alcoholic solution of 1.5% (v/v) 2,4-dinitrofluorobenzene was added to the samples and incubated for 4 h. Diethyl ether (1.0 ml) was added, and the samples were shaken and centrifuged at $2000 \times g$ for 20 min at room temperature. The residual aqueous phase containing derived glutathione was separated and analyzed by the HPLC-UV/VIS detection system (model LC-20AT and SPD-20A, Shimadzu). Separation of 5-carboxymethyl glutathione was carried out at room temperature with a flow rate of 1.2 ml/min. Chromatographic separation of derivatives was performed by injecting samples (10 μ l) of the aqueous phase onto a Spherisorb NH_2 5 μ m analytical column (Waters, Milford, MA, U.S.A.). Glutathione was subsequently detected using a UV detector at 365 nm. Glutathione derivatives (GSH and GSSG) were quantified in relation to the internal standard (cysteic acid).

2.14. Glutathione immunocytochemistry

Animals were sacrificed at 1 d after MA injection. They were deeply anesthetized with pentobarbital and perfused transcardially with 50 ml of 50 mM PBS, followed by 500 ml of a mixture of 1% paraformaldehyde and 2.5% glutaraldehyde in PBS. The rate of perfusion was 50 ml/min. Brain sections of 5- μ m thickness obtained from wild type (B6), p47 phox KO and PKC δ KO mice were placed on the same slide and processed for immunostaining. Tissue was adhered on poly-L-lysine-precoated coverslips, fixed in PBS-4 % paraformaldehyde (PFA), and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After saturation with PBS-1% BSA, tissues were incubated for 40 min with the primary antibody and incubated for 40 min with the secondary antibody. Rabbit anti-glutathione (1:100; a gift from Professor O.P. Ottersen, Laboratory of Molecular Neuroscience, Division of Anatomy, Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway), and goat anti-rabbit IgG H&L (Texas Red®) (1:300) (Life Technologies, Carlsbad, CA, U.S.A) were used as a secondary antibody. Images of samples were recorded using an FV1000 confocal microscope (Olympus, Tokyo, Japan).

The procedure for developing antibodies to glutathione was developed in the laboratory of one of the coauthors (Professor O.P. Ottersen) and is based on the use of glutaraldehyde as chemical fixating agent [61]. This principle was first developed for other small molecules such as GABA and glutamate [62]. The premise for the technique is indeed that glutathione is fixed by glutaraldehyde to the cells' protein matrix. In other words, the use of glutaraldehyde ensures that glutathione is irreversibly fixed in the cells and cell compartments where glutathione occurs. The fixation products thus formed retain the structural specificity of glutathione and can thus be identified by the appropriate antibodies. Obviously, being a small and diffusible molecule, glutathione needs to be immobilized prior to immunocytochemistry, and this is what is achieved when glutathione is chemically linked to the protein matrix by glutaraldehyde. Our antibodies have been used successfully by a

number of laboratories and we have documented previously that the antibodies discriminate between glutathione and other small molecules, including its component amino acids.

The glutathione antibody has been described elsewhere [60,61,63,64]. Antibodies raised against reduced form (GSH) also recognized the oxidized form (GSSG) [61]. Thus, attempts to raise antibodies that react with GSSG, but not with the reduced form, have not met with success [61,63,64]. It is likely, however, that the reduced form is responsible for the major share of the immunocytochemical signal, as the reduced form is known to predominate over the oxidized form [65,66].

2.15. Immunocytochemistry

Immunocytochemistry was performed as described previously [10]. 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 in 4% paraformaldehyde overnight. A series of every sixth section (35- μ m thickness, 210 μ m apart) from 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 a 48-h incubation with primary antibody against TH [1:500; Chemicon (EMD Millipore)] and Iba-1 (1:500, Wako Pure Chemical Industries, Chuo-ku, Osaka, Japan), sections were incubated with the biotinylated secondary antibody (1:1000; Vector Laboratories, Burlingame, CA, U.S.A.) for 1 h. The sections were then immersed in a solution containing avidin–biotin peroxidase complex (Vector Laboratories) for 1 h, and 3,3'-diaminobenzidine was utilized as the chromogen.

To examine TH-immunoreactivity, digital images were acquired at 4 $\times$ objective magnification using an Olympus microscope (BX51; Olympus) and a digital microscope camera (DP72; Olympus). ImageJ version 1.47 software (National Institutes of Health, Bethesda, MD, U.S.A.) was employed to measure the TH-immunoreactivity as described previously [67]. Briefly, the entire striatal region from each section was selected as the region of interest (ROI). Threshold values for hue (0–100), saturation (0–255), and brightness (175–255) were set in the “Adjust Color Threshold” dialog box, and then the mean density was measured.

2.16. Double-labeled immunocytochemistry

Brain sections of 5- μ m thickness obtained from mice were placed on the same slide and processed for immunostaining. Tissue was adhered on poly-L-lysine–precoated coverslips, fixed in PBS-4 % PFA, and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After saturation with PBS-1% BSA, tissues were incubated for 40 min with the primary antibody and incubated for 40 min with the secondary antibody as follows: primary antisera were as follows: mouse anti-neuronal nuclei (NeuN) (1:100) (Chemicon, Billerica, MA), anti-Glial fibrillary acidic protein (GFAP) (1:100) (Abcam, Cambridge, MA, U.S.A.), anti-Iba1 (1:100) (Abcam, Cambridge, MA, U.S.A.), and rabbit anti-glutathione (1:100) [60,61,63,64], rabbit anti-p-PKC delta (1:100) (Santa cruz, TX, USA) and rabbit anti-p47 phox (1:50) (Thermo Fisher scientific, MA, USA). Secondary antibodies were anti-mouse

IgG H&L (FITC) (1:200) (Abcam, Cambridge, MA, U.S.A.), anti-rabbit IgG H&L (Texas Red®) (1:300) (Life Technologies, Carlsbad, CA, U.S.A.), anti-rabbit IgG H&L (FITC) (1:200) (Abcam, Cambridge, MA, U.S.A.), anti-goat IgG H&L (Alexa Fluor® 405) (1:200) (Abcam, Cambridge, MA, U.S.A.), and anti-goat IgG H&L (Alexa Fluor® 546) (1:200) (Invitrogen, Carlsbad, CA, U.S.A.). Images of samples were recorded using an FV1000 confocal microscope (Olympus, Tokyo, Japan). To minimize bleed-through, each signal in double- or triple-stained samples was imaged sequentially. Images were processed in accordance with the Guidelines for Proper Digital Image Handling using ImageJ and/or Adobe Photoshop CS3 (Adobe, San Jose, CA, U.S.A.). Quantitative colocalization and statistical analysis are performed by FV1000 1.6 colocalization software (Olympus Tokyo, Japan) and ImageJ (free available on <http://rsbweb.nih.gov/ij/>).

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

Expression of the modifier and catalytic subunits of GCL (GCLc and GCLm, respectively) was assessed using quantitative RT-PCR to analyze mRNA levels, as described previously [55]. 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 the RNA to cDNA EcoDry Premix (Clontech, Palo Alto, CA, U.S.A.) with a 1-h incubation at 42°C. PCR amplification was conducted using 25 cycles 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, predicted product sizes, and GenBank accession numbers (where available) for the amplified genes were as follows: mouse GCLc (427 bp; #U85498.1), 5'-TTC CAA GCC TGC AGC ATA TC-3' (forward), and 5'-CAG ACT CGT TGG CAT CAT CC-3' (reverse); mouse GCLm (394 bp; #U95053.1), 5'-ATG GAG TTC CCA AAT CAG CC-3' (forward), and 5'-ATT GGG TTT TAC CTG TGC CC-3' (reverse); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 450 bp), 5'-ACC ACA GTC CAT GCC ATC-3' (forward), and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse). PCR products were separated on 2% agarose gels containing ethidium bromide, and visualized under ultraviolet light. Quantitative analysis of RNA was performed using PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat).

2.18. TUNEL staining

For TUNEL staining, a series of every sixth section (35-µm thickness, 210 µm apart) from striatum was selected. TUNEL staining was performed using the FragEL DNA fragmentation detection kit (QIA33; Calbiochem, La Jolla, CA, U.S.A.) according to the manufacturer's protocol [10,14,15]. Briefly, sections were permeabilized by incubation with 20 mg/ml proteinase K, and then incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. After immersion in the terminal deoxynucleotidyl transferase (TdT) equilibration buffer, sections were incubated with biotinylated deoxynucleotides and TdT enzyme. Sections were then immersed in streptavidin-peroxidase complex with diaminobenzidine tetrahydrochloride as the chromogen. Counterstaining was performed using methyl green, which was provided in the kit. Digital images from each quadrant of the striatum (dorsal-medial, dorsal-lateral, ventral-media, and ventral-lateral) were acquired [21,22] at 40 × objective magnification using an Olympus microscope (BX51; Olympus) and a digital microscope camera (DP72; Olympus). Cell counting was conducted blindly.

Apoptotic cells were identified based on the rounded, shrunken nature of the cytoplasm and nucleus, and on the intense staining of the nucleus. After counting, a mean value was obtained by averaging the counts of each quadrant from five sections for each animal [39,44].

2.19. Measurements of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)

Mice were sacrificed by cervical dislocation, and the brains were removed. The striatum was dissected, immediately frozen on dry ice, and stored at -70°C before 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 and its metabolites DOPAC and HVA were determined by HPLC coupled with an electrochemical detector, as described previously [10,67]. Supernatant aliquots (20 μL) were injected into an HPLC equipped with a C18 column with 3- μm particle size (Waters). The mobile phase was comprised of 26 mL of acetonitrile, 21 mL of tetrahydrofuran, and 960 mL of 0.15 M monochloroacetic acid (pH 3.0) containing 50 mg/L of EDTA and 200 mg/mL of sodium octyl sulfate. The amount of DA was determined by comparison of peak areas of tissue samples with standard, and was expressed in ng/g of wet tissue.

2.20. Statistical analyses

Data were analyzed using IBM SPSS ver. 21.0 (IBM, Chicago, IL, U.S.A.). One-way analysis of variance (ANOVA) was employed for statistical analyses. *Post-hoc* Fisher's least significant difference (LSD) pairwise comparison tests were then conducted. P-values < 0.05 were considered to be significant.

3. Results

3.1. MA-induced selective activation of PKC δ requires co-immunoprecipitation of PKC δ and p47phox in the striatum of wild-type (WT) mice

As the precise role of each PKC isoform in neurotoxicity induced by a toxic dose of MA remains elusive, we examined the striatal expression of PKC isoforms after MA administration. As shown in Supplementary Fig. S1A – D, expression of PKC α , βI , βII , and ζ was not significantly changed at 15 min, 30 min, 1 h, 2 h, 6 h, 1 d, 3 d, and 7 d after MA administration in WT mice. However, significant increases in PKC δ expression were noted from 15 min to 7 d after MA administration (Fig. 1A). PKC δ phosphorylations were significantly increased 15 min, 30 min, 1 h, 2 h, and 4 h after MA administration. The increase was most pronounced 15 min after the MA administration (Fig. 1B). In contrast, MA-induced cleaved PKC δ expression was significantly increased 2 h, 4 h, 6 h, and 1 d later. The most significant increase of the cleaved PKC δ was observed 1-d post-MA (Fig. 1B). In order to assess the binding of PKC to p47phox *in vivo*, we performed immunoprecipitation experiments. PKC δ co-immunoprecipitates with p47phox in the striatum of treated with MA. The association occurred rapidly at 30 min and 1 h, and was maximal after 30 min (Fig. 1C).

3.2. Effects of pharmacological or genetic inhibition of PKC δ on the phosphorylation and membrane translocation of p47phox induced by MA

We, therefore, proceeded to test the potential involvement of PKC δ in PHOX activity and, more specifically, in p47phox phosphorylation. Phosphorylation of p47phox was significantly increased 30 min, 1 h, 2 h, 4 h, and 6 h after MA treatment. The most significant phosphorylation of p47phox was observed 30 min post-MA (Fig. 1D). Rottlerin, a drug previously shown to be a selective inhibitor of PKC δ enzyme activity [68], caused an inhibition of p47phox phosphorylation (Fig. 1F). Consistently, genetic depletion of PKC δ [by utilizing knockout (KO) mice] also significantly inhibited p47phox phosphorylation induced by MA (Fig. 1F). These results suggest that PKC δ regulates the phosphorylation of p47phox. Because phosphorylation of p47phox regulates its translocation [69–71], we tested the effect of rottlerin on the translocation of p47phox. Membrane translocation of p47phox was significantly increased 1 h, 2 h, 4 h, 6 h, 1 d, and 3 d after MA administration (Fig. 1E). As MA-induced translocation of p47phox was most significant 2 h later (Fig. 1E), we examined the effects of rottlerin in response to this translocation at that time (Fig. 1G). Rottlerin inhibited the membrane translocation of p47phox induced by MA. Consistently, PKC δ KO mice protected membrane translocation of p47phox induced by MA (Fig. 1G).

3.3. Effects of rottlerin on the changes in the PHOX activity and ROS formation induced by MA in the striatum of p47phox knockout (KO) and WT mice in comparison with PKC δ KO mice

To determine whether inhibiting the phosphorylation and translocation of p47phox affects oxidative stress, we examined the effect of rottlerin or PKC δ KO on PHOX activity and synaptosomal ROS production induced by MA in the striatum of mice. Initial increase in PHOX activity was observed 30 min after MA administration. This increase remains elevated until 1 d after MA administration. This increase of PHOX activity was most evident 1 h and 2 h-post MA (Fig. 2A). Similarly, ROS was significantly increased 30 min, 1 h, 2 h, 4 h, 6 h, and 1 d after MA. The increase in ROS was most prominent 2 h and 4 h after MA administration (Fig. 2B). Thus, we focused on the 2 h time-point, and then examined the effects of p47phox KO, rottlerin, and PKC δ KO in response to PHOX activity or ROS formation induced by MA.

The data in Fig. 2C and D showed that rottlerin inhibits PHOX activity and ROS production by activated MA in WT mice. Either p47phox KO or PKC δ KO attenuated PHOX activity (Fig. 2C) and ROS formation (Fig. 2D) induced by MA. Rottlerin did not exhibit any additional protective activity in response to attenuation by p47phox KO (Fig. 2C and D). Taken together, our results indicate that PKC δ regulates PHOX activity through its effects on p47phox phosphorylation and membrane translocation.

3.4. Effects of rottlerin on the changes in Nrf-2 nuclear translocation and Nrf-2 DNA binding induced by MA in the striatum of p47phox knockout (KO) and WT mice in comparison with PKC δ KO mice

We next examined whether rottlerin-mediated antioxidant potential is positively regulated by the expression of Nrf2. As presented in Fig. 3, we examined whether nuclear Nrf2 translocation alters against MA insult. In the absence of MA, Nrf2 was found in high

amounts in the cytosol in WT mouse striatum. However, MA-induced initial increases in Nrf2 nuclear translocation were observed at 30 min post-MA. This increase remains elevated 7 d later (Fig. 3A). The maximal increase was observed 6 h after MA administration, while the minimal increase was noted 1 d after MA administration (Fig. 3A). Thus, we focused on 1-d post-MA for further studies. Rottlerin or PKC δ KO significantly attenuated decreased Nrf2 nuclear translocation 1-d post-MA. Genetic inhibition of p47 $phox$ (by utilizing p47 $phox$ KO) significantly protected a decrease in Nrf2 nuclear translocation induced by MA. However, rottlerin did not show any additive effects in response to protective activity mediated by p47 $phox$ KO (Fig. 3C). The profile of nuclear Nrf2 expression paralleled that of Nrf2 DNA-binding activity (Fig. 3B and D).

3.5. Effects of rottlerin on the changes in GCLc- and GCLm-mRNA, GSH- and GSSG-levels, and glutathione-immunoreactivity induced by MA in the striatum of p47 $phox$ knockout (KO) and WT mice in comparison with PKC δ KO mice

We next examined whether rottlerin, PKC δ KO, or p47 $phox$ KO, would up-regulate GCLc and GCLm mRNA levels, and GSH synthesis. Significant increases in GCLc and GCLm mRNA expression were observed 4 h, 6 h, 3 d, and 7 d after MA administration in the striatum of WT mice, whereas significant decreases in the GCLc and GCLm mRNA expression were noted 1 d after MA administration (Fig. 4A and B). MA-induced changes in GSH and GSSG level were shown in Fig. 4 C and D. GSH level appeared to be increased in the early stage, 30 min after MA treatment. However, GSH level was significantly decreased 1 d after MA treatment, although this decrease appeared to return to near the control level 3 d and 7 d later (Fig. 4C). Consistently, GSSG was significantly increased 1 d after MA administration (Fig. 4D). Thus, we concentrated on 1-d post-MA for further studies. Rottlerin or PKC δ KO significantly attenuated MA-induced decreases in GCLc and GCLm mRNA expressions (Fig. 4E and F), and GSH level (Fig. 4G) and glutathione-immunoreactivity (Fig. 4I), whereas attenuated MA-induced increase in GSSG level (Fig. 4H). Consistently, genetic inhibition of p47 $phox$ (by utilizing p47 $phox$ KO) significantly protected these alterations in the GCLc, and GCLm expressions, as well as GSH and GSSG levels and glutathione-immunoreactivity. However, rottlerin did not exhibit any additive effects in response to protective potentials mediated by p47 $phox$ KO (Fig. 4B, D, F, H, and I).

3.6. Effects of BSO, an inhibitor of glutathione biosynthesis, on the pharmacological activity of rottlerin against changes in Iba-1-, GFAP- or NeuN-expression induced by MA in the striatum of p47 $phox$ knockout (KO) and WT mice in comparison with PKC δ KO mice

It was recognized that the nigrostriatal area is more sensitive to the GSH-depleting effects of BSO than other parts of brain, including the cortex and the limbic system [72,73]. GSH depletion might result in cellular degeneration in the nigrostriatal system [74]. Treatment with BSO resulted in significant decreases in glutathione-IR and GSH levels (Supplementary Fig. S3).

As shown in Fig. 5A, MA-induced initial increase in Iba-1 expression was observed 30 min later. This Iba-1 expression remains significantly elevated 7 d later. Since MA-induced maximal increase of Iba-1 expression was most pronounced 1-d post MA, we focused on

this time for further study (Fig 5D). In contrast, treatment with MA results in significant decreases (6 h, 1 d, 3 d, and 7 d post-MA) in NeuN expression (Fig. 5B) and in significant increases (from 15 min to 7 d post-MA) in GFAP expression (Fig. 5C). Hence, the MA-induced increasing pattern of GFAP is different from that in Iba-1 expression (Fig. 5A and C). It is important to note that these changes in Iba-1-, GFAP-, and NeuN-expression precede inactivation of Nrf2-dependent GCL pathway.

MA-induced increase in Iba-1 expression was significantly attenuated by rottlerin, PKC δ KO, or p47 $phox$ KO. BSO significantly counteracted against the reduction in Iba-1 expression by p47 $phox$ KO, rottlerin, or PKC δ KO. Results from Western blot analysis (Fig. 5D) support those from immunocytochemical analysis (Fig. 5G). However, neither NeuN-expression nor GFAP expression was affected by BSO in the presence of MA. Therefore, these results suggest that depletion of p47 $phox$ or PKC δ is critical for attenuating MA neurotoxicity via inhibition of microglia by GSH modulation.

In addition, significant microglial activation was observed 1 d after MA treatment, as revealed by cell skeleton analysis and cell size analysis (Supplementary Fig. S4). Consistently, the finding of Fig. 5D and G paralleled that of Supplementary Fig. S5.

3.7. Glutathione-, phospho-PKC δ -, phospho-p47 $phox$ -immunoreactivities are co-localized with NeuN-labeled neurons or Iba-1 labeled microglial cells in the striatum of WT mice

Since little is currently known regarding whether MA treatment alters glutathione immunodistribution in the brain of mice, immunocytochemical analysis was carried out after MA treatment to characterize the cellular location of glutathione. Glutathione-immunostained sections from the saline-treated group (without MA) showed a moderate glutathione-immunoreactivity in striatum. Double-labeling immunocytochemical analysis was conducted 15 min, 30 min, 4 h, and 1 d post-MA (Fig. 6A and B). A little glutathione-immunoreactivity (glutathione-IR) was present in NeuN-positive cells or Iba-1-positive cells in the striatum of saline-treated mice, indicating that glutathione-IR colocalizes with neurons or microglia (Fig. 6A and B). Glutathione-IR in the neurons was significantly increased ($P < 0.05$ vs. saline) 30 min post-MA. This increase in glutathione-IR returned to near saline level 4 h and 1 d post-MA (Fig. 6A). Initial increase ($P < 0.05$ vs. saline) of glutathione-IR in the Iba-1 labeled microglial cells was observed 15 min post-MA. This microglial glutathione-IR was maximally induced ($P < 0.01$ vs. saline) 4 h (> 30 min) post-MA. This microglial glutathione-IR remains elevated ($P < 0.05$) 1 d later. Unexpectedly, glutathione-IR was not localized in the GFAP-labeled astrocytes 30 min and 1 d post-MA (Supplementary Fig. S6).

The induction of PKC δ in neurons and microglia suggests that PKC δ could be the major mediator or modulator of proapoptotic and proinflammatory responses to neurotoxic insults [75]. Interestingly, increased expression of PKC δ within Iba-1-labeled reactive microglia was observed in the ventral mesencephalon of post-mortem of Parkinsonian brains [76].

Here, we conducted double-labeled immunocytochemical assay to understand whether MA-induced neuronal and microglial stimulations require phosphorylation of PKC δ . A little phospho-PKC δ -immunoreactivity (p-PKC δ -IR) was observed in NeuN-labeled neurons or

in Iba-1-labeled microglia in the striatum of saline-treated mice. MA treatment facilitated the co-localization. p-PKC δ -IR in neurons and microglia was maximally increased ($P < 0.01$ vs. saline) 15 min post-MA, respectively. The increase in p-PKC δ -IR in neurons and microglia lasted for 4 h. This increase in neuronal or microglial p-PKC δ -IR returned to near saline level 1 d post-MA (Fig. 7 A and B).

It is known that a PHOX is thought to function in microglial cells of the CNS. p47phox, an essential component of the phagocyte PHOX, is required for superoxide anion release from microglia [77]. In this study, a very little phospho-p47phox-immunoreactivity (p-p47phox-IR) in NeuN-labeled neurons presented in the striatum of mice. MA treatment significantly induced ($P < 0.05$ vs. saline) p-p47phox-IR in the NeuN-labeled neurons 30 min later. After that, this co-localization returned to near saline level (Fig. 8A). Similarly, a little p-p47phox-IR was observed in the Iba-1-labeled microglia in the striatum of saline-treated mice. However, MA treatment significantly increased this co-localization 30 min ($P < 0.01$ vs. saline), 4h ($P < 0.05$ vs. saline), and 1 d ($P < 0.05$) post-MA. Maximal co-localization was noted 30 min post-MA (Fig. 8B).

3.8. Effects of BSO, an inhibitor of glutathione biosynthesis, on the protective activity of rottlerin, PKC δ antisense oligonucleotide (PKC δ ASO), or p47phox antisense oligonucleotide (p47phox ASO) against MA-induced increase in TUNEL-positive cells in the striatum of Taconic ICR mice

As we [10,14,15,47] and others [21,22] have reported, it is well-recognized that Taconic ICR mice are sensitive to MA-induced TUNEL-positive reaction. It was well-recognized that 1-d post MA is most sensitive to TUNEL-positive reaction [10,14,15,21,22,47]. In the absence of MA, TUNEL-positive cells were barely observed. They were significantly increased ($P < 0.01$) 1 d after a toxic dose of MA (35 mg/kg. i.p.). This significant increase (with p47phox SO or vehicle) in Taconic ICR mice was significantly inhibited ($P < 0.01$) by rottlerin, PKC δ ASO, or p47phox ASO in Taconic ICR mice. This inhibition was counteracted by BSO (Fig. 9).

3.9. Effects of BSO, an inhibitor of glutathione biosynthesis, on the pharmacological activity of rottlerin against proapoptotic changes induced by MA in the striatum of p47phox knockout (KO) and WT mice in comparison with PKC δ KO mice

The PI3K/Akt signaling is a critical mediator of cell survival. In the presence of survival factors, activated Akt phosphorylates specific residues of pro-apoptotic proteins, such as Bad, suppressing their apoptotic activity and thereby promoting cell survival. The interaction of phosphorylated Bad with 14-3-3 protein prevents the association of Bad with Bcl-xL at the mitochondrial membrane [78]. Thus, 14-3-3 protein acts as an anti-apoptotic factor by sequestering pro-apoptotic molecules, such as Bad. Because MA-induced TUNEL-positive reaction [10,14,15,21,22] was most pronounced 1 d after MA treatment, we simultaneously examined whether the mechanism by which rottlerin, PKC δ KO, or p47phox KO counters MA-induced damage, depends on the GSH system that inhibits the MA-induced expression of pro-apoptotic factors. We examined the effect of MA treatment on the activation of Akt (by phosphorylation at Ser473 to create p-Akt) and the deactivation of Bad (by phosphorylation at Ser136 to create p-Bad) in WT mice-treated with rottlerin, or p47phox

KO mice (Fig. 10). Treatment with MA significantly decreased levels of p-Akt and p-Bad in the striatum of WT mice. Treatment with rottlerin increased p-Akt and p-Bad levels measured 1 d after MA in WT mice. Administration of BSO significantly inhibited this effect of rottlerin on levels of p-Akt and p-Bad in WT mice. Rottlerin treatment did not show any additional positive effects against the activity mediated by p47*phox* KO (Fig. 10A and B).

Using immunoprecipitation analysis, we examined the effect of MA treatment on the interaction between p-Bad and 14-3-3 protein in p47*phox* KO or PKC δ KO mice (Fig. 10C). MA treatment decreased this interaction in both WT- and p47*phox* KO-, and in both WT- and PKC δ KO mice, but this effect was more pronounced in WT mice. Treatment with rottlerin increased the p-Bad/14-3-3 protein interaction in WT mice, treated with MA (Fig. 10C). This effect of rottlerin on the p-Bad/14-3-3 protein interaction was inhibited by BSO (Fig. 10C). Similarly, MA treatment decreased the expression of the anti-apoptotic proteins Bcl-xL and Bcl-2 in both WT- and p47*phox* KO-, and in both WT- and PKC δ KO mice, but this effect was more pronounced in the WT than the p47*phox* KO or PKC δ KO mice, respectively. Treatment with rottlerin increased the expression of Bcl-2 and Bcl-xL in WT mice in the presence of MA (Fig. 10D and E). This effect of rottlerin for both Bcl-2 and Bcl-xL was inhibited by BSO (Fig. 10D and E). As presented in Fig. 10F, G and H, MA treatment increased levels of the pro-apoptotic factors Bax, cleaved PKC δ and cleaved caspase-3 in both WT- and p47*phox* KO-, and in both WT- and PKC δ KO mice, but this effect was more evident in the WT than the p47*phox* KO or PKC δ KO mice, respectively. This increase was more prominent in WT mice. Treatment with rottlerin attenuated the MA-induced increase in Bax, cleaved PKC δ , and cleaved caspase-3 levels. Treatment of rottlerin did not produce any additional protective effects against the activity mediated by p47*phox* KO. The attenuating effect of rottlerin, p47*phox* KO, or PKC δ KO was inhibited by BSO, respectively. These results suggest that the endogenous GSH system plays an essential role in exerting anti-apoptotic effects mediated by rottlerin, PKC δ KO, or p47*phox* KO against MA insult.

3.10. Effects of BSO, an inhibitor of glutathione biosynthesis, on the protective activity of rottlerin against MA-induced dopaminergic impairments

MA-induced initial decrease in TH expression was observed 2 h later, indicating that dopaminergic loss precedes inactivation of Nrf2-dependent system. However, the most significant decrease was noted 1-d post-MA (Fig. 11A). Although TH expression appears to recover after 1 d post-MA, it still remains reduced 7 d later. MA-induced decrease in TH expression apparently supports MA-induced decrease in dopamine (Fig. 11B). Consistently, MA-induced increase in dopamine turnover rate was observed (Fig. 11C). Either p47*phox* KO or PKC δ KO significantly attenuated MA-induced decrease in TH expression. Rottlerin did not exhibit additional positive effects against the activity mediated by p47*phox* KO. Rottlerin-mediated protective effects were counteracted by BSO (Fig. 11D). Results of Western blot analysis of TH were clearly comparable to those of immunocytochemical analysis of TH (Supplementary Fig. S7), and to those of dopamine levels (Fig. 11E). Consistently, MA-induced increase in dopamine turnover rate in WT mice was attenuated by p47*phox* KO or PKC δ KO (Fig. 11F). Although rottlerin significantly attenuated dopamine

turnover rate in WT mice, it did not significantly alter attenuation by p47^{phox} KO. Positive effects mediated by p47^{phox} KO, rottlerin, or PKC δ KO were counteracted by BSO (Fig. 11F).

4. Discussion

p47^{phox} is an essential component of PHOX, and phosphorylation of p47^{phox} is associated with activation of the enzyme. Earlier investigation suggested that interaction occurs at the catalytic site of PKC and the C-terminus of p47^{phox}, and is specific for p47^{phox} [2]. We showed that PKC δ co-immunoprecipitates with p47^{phox} in the striatum of mice-treated with MA and that this association occurs rapidly (Fig. 1C), suggesting that PKC δ expression is required for striatal PHOX activity through the regulation of phosphorylation and translocation of p47^{phox}. In line with the current study, earlier *in vitro* studies with PMA-stimulated neutrophils or B lymphocytes have also indicated that activation of PHOX requires phosphorylation of p47^{phox}, which occurs before its translocation [79–81].

The current study also indicates that inhibition of phosphorylation prevents translocation, and blocks PHOX activity and ROS formation. In addition to our data, which corroborate previous findings that PKC is involved in stimulus-induced regulation of the PHOX of phagocytic cells, we report that a specific PKC isoform regulates p47^{phox} phosphorylation and p47^{phox} translocation induced by MA. Importantly, PKC is a major intracellular receptor for 12-O-tetradecanoylphorbol-13-acetate (TPA) that can activate classical and novel PKC isoforms [82], such as PKC δ , which is an oxidative stress-sensitive kinase whose activation induces apoptotic cell death via inflammatory apoptosis in MA neurotoxicity [10,14,15,30].

It was demonstrated that PKC is among a group of cell-signaling molecules that are sensitive targets for redox modification [83]. In terms of redox-stimulated signaling, it is important to note that significant overlap exists between PKC-mediated and oxidant-induced signaling responses [83]. Importantly, it is recognized that PKC δ (out of PKC isoforms) activity might be subject to redox regulation by endogenous molecule GSH [84]. Reduction of GSH levels and intracellular oxidative stress differentially influences the functional activity of novel PKC δ . GSH loss in neuroblastoma cells leads to increased PKC δ activity and ROS production, and these increases were protected by rottlerin [85,86]. Therefore, intracellular oxidative imbalance resulting from experimental GSH depletion can selectively influence the PKC δ -dependent signal transduction system [31].

Here, we observed that MA treatment increased PHOX activity and ROS formation, and MA treatment decreased GSH (consistently increased GSSG) and glutathione (GSH and GSSG)-immunoreactivity. These oxidative burdens were significantly protected by inhibition of PKC δ or p47^{phox}, and more importantly, MA treatment caused a persistent disruption of glutathione homeostasis in WT. However, this phenomenon was not evident in PKC δ KO or p47^{phox} KO mice, suggesting that PKC δ or p47^{phox} is a critical mediator for glutathione modulation against MA insult. In addition, it is possible that the functional regulation of PHOX activity by PKC δ is corroborated by our results showing that inhibition of PKC δ also blocks ROS formation, as well as impairment in glutathione system.

Under oxidative stress, Nrf2 modulates cytoplasmic responses to oxidative stress [87] through the transcriptional activation of genes involved in GSH synthesis, including those encoding GCLc and GCLm [88–90]. Although it seems highly probable that early neurotoxic signals (i.e., increases in p-PKC δ , p-p47phox, PHOX activity, ROS, lipid peroxidation, protein oxidation and dopamine turnover rate) induced by MA facilitate a compensatory mechanism in which Nrf2 moves into the nucleus and increases GCLc, and GCLm expression, these signals might be a prerequisite for inactivating Nrf2-GCL pathway. In line with current study, Jayanthi et al. [91] reported Nrf2 activation in the striatum of rats treated with MA. Granado et al. [92] observed that Nrf2 deficient mice are more sensitive than WT to MA-induced striatal damage. They also suggested Nrf2 activation is part of a defensive response [92]. Interestingly, Jayanthi et al. [91] demonstrated MA-induced activation of the endoplasmic reticulum (ER) stress response. Therefore, based on their data [91], it is plausible that activation of ER stress response might lead to induction of Nrf2 in response to MA insult, although it remains to be explored.

In this study, we showed the significance of PKC δ and p47phox signaling in regulating microglial activated response, and its relationship to dopaminergic neurotoxicity induced by MA. We suggest that early induction of p-PKC δ in the microglial cells triggers the activation of p47phox, which is accompanied by initial neurotoxic signals, and that early interaction of p-PKC δ and p-47phox are integral to sustained microglial activation and dopaminergic neurodegeneration.

In this study, MA-induced activated microglial cells also possess glutathione-IR, suggesting that the glutathione-IR may mainly be a reflection of the necessity for self-protection against oxidative burdens produced by microglia in the presence of MA. Indeed, microglia in culture, maintain a higher concentration of GSH than either astrocytes or neurons [93], which underlies their considerable resistance to oxidative stress.

There are several instances in which changes in the GSH pool alter the extent of microglial activation. Lee et al. [94] demonstrated that depletion of GSH in human microglial cells with BSO induces oxidative stress and an inflammatory response. Therefore, we agree that the GSH pool plays a critical role in neuroinflammation linked to neurotoxic disorders, and, thus, represents a potential target for treatment. However, it remains to be elucidated how MA-induced early inductions of p-PKC δ , p-47phox and glutathione-IR modulate interaction between neurons and microglial cells, and how interactive signaling network of p-PKC δ , p-47phox and glutathione in the reactive microglial cells contributes to MA-induced dopaminergic neurodegeneration.

In contrast, we reported that hippocampal glutathione-immunoreactivity was induced in the proliferating astroglia-like cells by 3 weeks after treatment with trimethyltin (TMT) in rats, although we applied the same primary antisera of rabbit anti-glutathione [60]. Double-label immunostaining for glutathione and GFAP revealed that glutathione-immunoreactivity and GFAP-immunoreactivity were localized in the same cell, suggesting that glutathione was specifically induced in the astrocytes in the hippocampus against TMT-induced neuronal injury [60]. As ascorbate treatment significantly attenuated the proliferation of the glutathione-immunoreactive astrocytes, we suggested that glutathione-positive reactive

astrocytes may take part in a compensative mechanism against the TMT-induced elimination of glutathione-immunoreactive pyramidal neurons owing to neuronal degeneration [60]. Here, we confirmed that the cellular mobilization of glutathione-immunoreactivity induced by TMT is different from that induced by MA. Although it might be plausible that the different neurotoxic mechanism might require complex/different signaling pathways between neuronal, astroglial and microglial interactions in the different brain areas (e.g., hippocampus and striatum), this phenomenon remains to be clarified.

Interestingly, treatment of WT mice with rottlerin significantly attenuated the oxidative burdens induced by MA. It also attenuated the MA-induced inhibition of pro-apoptotic signaling: it enhanced p-Akt, Bcl-2 and Bcl-xL expression, Bad phosphorylation, and 14–3–3/p-Bad complex formation, and inhibited Bax expression, PKC δ cleavage, and caspase-3 cleavage. It also significantly reduced MA-induced pro-apoptotic changes in the striatum of WT mice, an effect that was inhibited by the BSO. Thus, inhibition of PKC δ /p47 $phox$ or PKC δ -dependent p47 $phox$ activation provided a significant protection against MA-induced pro-apoptotic degeneration through a mechanism involving activation of Nrf2-dependent GSH induction.

As the p-Bad/14–3–3 interaction is necessary for suppression of cell death [78,95], we proposed that the neuroprotective activity mediated by inhibition of PKC δ -dependent p47 $phox$ activation might result, at least in part, from the alterations in the formation of the p-Bad/14–3–3 complex. In addition, our data clearly suggest that inhibition of PKC δ or p47 $phox$ protects neuronal cells by inhibiting caspase-3 cleavage. Thus, either treatment might act as a multifunctional neuronal factor, functioning in neuronal survival and in neuroprotection against MA insult.

Under our experimental conditions, inhibition of PKC δ or p47 $phox$ markedly increased levels of p-Akt, p-Bad, 14–3–3 protein-associated p-Bad, and Bcl-xL (Bcl-2), and decreased levels of Bax and cleaved caspase-3. The pro-survival effects were significantly inhibited by treatment with BSO, suggesting that maintenance of the interaction between p-Bad and 14–3–3 through the glutathione defense mechanism is critical for pro-survival effects mediated by inhibition of PKC δ -dependent p47 $phox$ activation.

We [10,44,67] and others [96] proposed possible links between MA neurotoxicity and microglial activation, including early changes in microglial-associated gene expression. It was observed that MA-induced activation of microglia selectively occurs in the area of TH-fiber loss of the striatum (Supplementary Fig. S8). Our results are consistent with previous findings [97], and suggest that microglial reactivity remains a specific marker for acute damage to DA terminals following a neurotoxic regimen of MA.

In addition, MA treatment significantly increased the mRNA expression of M1 phenotypic markers (CD16, CD32, and CD86), suggesting that microglia after MA treatment existed primarily in the classically activated state [10,67], which is proinflammatory. Previous work demonstrated that reactive microglia [98,99] increased in the brains of human MA abusers. Importantly, activation of caspases regulates microglia activation through a PKC δ -dependent pathway [100]. Thus, our results suggest that neuroprotection by inhibition of PKC δ -

dependent p47^{phox} activation is, at least in part, mediated by its anti-inflammatory properties. MA-induced pro-apoptosis (i.e., TUNEL-positive cells) peaks at 24 h [15,21,22], while DA terminal markers (TH, DAT, and VMAT2) remain lower for 14 d after the final MA dose [14,15], indicating that MA-induced PKC δ -dependent p47^{phox} activation, oxidative stress, followed by microglial activation and pro-apoptosis, precedes DA terminal toxicity.

In summary, we demonstrate for the first time that PKC δ specifically regulates the function of PHOX in MA-induced neurotoxicity, and that PKC δ -dependent p47^{phox} activation, oxidative stress, imbalance of Nrf-2-dependent glutathione synthesis, microglial activation/alteration, and pro-apoptosis induced by MA contribute to dopaminergic toxicity, suggesting that PKC δ is an important regulator of p47^{phox} phosphorylation and translocation in dopaminergic neurotoxicity induced by MA. Future studies are needed to precisely characterize whether PKC δ is an immediate upstream kinase of p47^{phox}, to elucidate the significance in the interactive modulation of p-PKC δ -IR, p-47^{phox}-IR, and glutathione-IR in the microglial cells, and to define the role of PKC δ in regulating PHOX phosphorylation and activity in other dopaminergic neurodegenerative disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ANOVA	analysis of variance
ASO	antisense oligonucleotide
BSO	buthionine-sulfoximine
DA	dopamine
DAT	dopamine transporter
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
GCLc	γ -glutamylcysteine ligase catalytic subunit
GCLm	γ -glutamylcysteine ligase modifier subunit
GFAP	glial fibrillary acidic protein
GSH	glutathione
GSSG	glutathione disulfide
HVA	homovanillic acid
Iba-1	ionized calcium binding adaptor molecule 1
KO	knockout
MA	methamphetamine
PBS	phosphate-buffered saline
PD	Parkinson's disease
PFA	para-formaldehyde
PHOX	NADPH oxidase
PKC	protein kinase C
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
RT-PCR	reverse transcription and polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SO	sense oligonucleotide
TH	tyrosine hydroxylase
TUNEL	terminal deoxynucleotidyl transferase dUDP nick end labeling
WT	wild-type

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Highlights

- Interplay between PKC and PHOX *in vivo* in the neurotoxic condition remains unclear
- PKC δ co-immunoprecipitated with p47 $phox$ and facilitated the potential of p47 $phox$
- Methamphetamine-induced Nrf-2 system was mitigated by inhibition of p47 $phox$ or PKC δ
- BSO potentiated microgliosis and apoptotic changes, leading to dopaminergic losses
- PKC δ is a critical regulator for p47 $phox$ activation induced by methamphetamine

Fig. 1.

MA-induced changes in PKCδ (A), phospho-PKCδ, cleaved PKCδ expression (B), co-immunoprecipitation of PKCδ and p47*phox* (C), phospho-p47*phox* (D), effects of PKCδ inhibition against MA-induced maximal increases in phospho-p47*phox* (E), membrane translocation of p47*phox* (F), and membrane translocation of p47*phox* (G) in the striatum of mice. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rottlerin 3 μg, i.c.v. WT = wild-type mice. Each value is the mean ± SEM of 6 animals. *P < 0.05 vs. Sal. **P < 0.01 vs. Sal or WT with Sal/Veh. #P < 0.05, ##P < 0.01 vs. WT with MA/Veh (one-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 2.

MA-induced increases in PHOX activity (A) and reactive oxygen species (ROS) (B) in the striatum of WT mice, and effects of PKC δ inhibition or p47 $phox$ inhibition against MA-induced maximal increases in PHOX activity (C) and ROS (D) in the striatum of p47 $phox$ knockout (KO) and WT mice in comparison with PKC δ KO mice. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rottlerin 3 μ g, i.c.v. WT = wild-type mice. Each value is the mean $\pm$ SEM of 6 animals. *P < 0.05 vs. Sal. **P < 0.01 vs. Sal or WT with Sal/Veh. #P < 0.05 vs. WT with MA/Veh (one-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 4.

MA-induced alterations in γ -glutamylcysteine ligase catalytic subunit (GCLC; A), γ -glutamylcysteine ligase modifier subunit (GCLM; B), GSH (C), GSSG (D) in the striatum of WT mice, and effects of rotterlin on the alterations in the GCLC (E), GCLM (F), GSH (G), GSSG (H), and glutathione-immunoreactivity (I) induced by MA in the striatum of p47^{phox} knockout (KO) and WT mice in comparison with PKC δ KO mice. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rotterlin 3 μ g, i.c.v. WT = wild-type mice. Each value is the mean $\pm$ SEM of 6 animals. *P < 0.05 vs. Sal or WT with Sal/Veh. #P < 0.05 vs. WT with MA/Veh (one-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 100 μ m.

Fig. 5.

MA-induced changes in Iba-1 (A), NeuN (B) and GFAP (C) expression in the striatum of WT mice, and effects of buthionine-sulfoximine (BSO), an inhibitor of glutathione biosynthesis on the pharmacological activity of rottlerin against MA-induced changes in Iba-1 (D), NeuN (E), GFAP (F) expression and Iba-1-immunoreactivity (G) in the striatum of p47^{phox} knockout (KO) and WT mice in comparison with PKC δ KO mice. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rottlerin 3 μ g, i.c.v. WT = wild-type mice. Each value is the mean $\pm$ SEM of 6 animals. *P < 0.05 vs. Sal, p47 KO with Sal/Veh, WT with Sal/Veh, or PKC δ KO with Sal. **P < 0.01 vs. Sal or WT with Sal/Veh. #P < 0.05, ##P < 0.01 vs. WT with MA/Veh. &P < 0.05, &&P < 0.01 vs. p47 KO with MA/Rot/Sal, WT with MA/Rot/Sal, or PKC δ KO with MA/Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 100 μ m.

Fig. 6.

Glutathione-immunoreactivity in the presence of MA was co-localized in the NeuN-labeled neurons (A) and Iba-1-labeled microglia (B). Arrows indicate co-localization in the NeuN-labeled neurons or Iba-1-labeled microglia. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Each value is the mean $\pm$ SEM of 4 animals. * $P < 0.05$ vs. Sal, ** $P < 0.01$ vs. Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 40 μ m.

Fig. 7.

Phospho-PKC δ (p-PKC δ)-immunoreactivity in the presence of MA was co-localized in the NeuN-labeled neurons (A) and Iba-1-labeled microglia (B). Arrows indicate co-localization in the NeuN-labeled neurons or Iba-1-labeled microglia. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Each value is the mean $\pm$ SEM of 4 animals. * $P < 0.05$ vs. Sal, ** $P < 0.01$ vs. Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 40 μ m.

Fig. 8.

Phospho-p47^{phox} (p-p47)-immunoreactivity in the presence of MA was co-localized in the NeuN-labeled neurons (A) and Iba-1-labeled microglia (B). Arrows indicate co-localization in the NeuN-labeled neurons or Iba-1-labeled microglia. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Each value is the mean $\pm$ SEM of 4 animals. * $P < 0.05$ vs. Sal, ** $P < 0.01$ vs. Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 100 μ m.

Fig. 9.

Effects of buthionine-sulfoximine (BSO), an inhibitor of glutathione biosynthesis, on the activity of rottlerin against MA-induced TUNEL positive cells in the striatum of Taconic ICR mice-treated with p47*phox* sense oligonucleotide (SO) or p47*phox* antisense oligonucleotide (ASO), and with PKCδ SO or PKCδ ASO. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rottlerin 3 μg, i.c.v. Each value is the mean ± SEM of 6 animals. **P < 0.01 vs. Sal/p47 SO/Sal or Sal/ PKCδ SO/Sal. ##P < 0.01 vs. MA/p47 SO/Veh/Sal or MA/PKCδ SO/Sal. &&P < 0.01 vs. MA/p47 SO/Rot/Sal, MA/p47 ASO/Veh/Sal, MA/p47 ASO/Rot/Sal, or MA/PKCδ ASO/Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons). Scale bar = 100 μm.

Fig. 10.

Effects of buthionine-sulfoximine (BSO), an inhibitor of glutathione biosynthesis, on the activity of rottlerin against MA-induced changes in p-Akt (A), p-Bad (B), 14-3-3/p-Bad binding (C), Bcl-2 (D), Bcl-xL (E), Bax (F), cleaved PKCδ (G), and cleaved caspase-3 (H) in the striatum of p47^{phox} knockout (KO) and WT mice in comparison with PKCδ KO mice. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rottlerin 3 μg, i.c.v. WT = wild-type mice. Each value is the mean ± SEM of 6 animals. **P < 0.01 vs. WT with Sal/Veh. #P < 0.05 vs. WT with MA/Veh. &P < 0.05 vs. p47 KO with MA/Rot/Sal, WT with MA/Rot/Sal, or PKCδ KO with MA/Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 11.

MA-induced alterations in tyrosine hydroxylase (TH) expression (A), dopamine level (B), and dopamine turnover rate (C), and effects of buthionine-sulfoximine (BSO), an inhibitor of glutathione biosynthesis, on the activity of rottlerin against decreases in the TH expression (D), dopamine level (E), and increase in dopamine turnover rate (F) induced by MA in the striatum of p47*phox* knockout (KO) and WT mice in comparison with PKCδ KO mice. Sal = saline. MA = methamphetamine 35 mg/kg, i.p. Veh = vehicle [10% (v/v) DMSO]. Rot = rottlerin 3 μg, i.c.v. WT = wild-type mice. Each value is the mean ± SEM of 6 animals. *P < 0.05 vs. Sal. **P < 0.01 vs. Sal or WT with Sal/Veh. #P < 0.05 WT with MA/Veh. &P < 0.05 vs. p47 KO with MA/Rot/Sal, WT with MA/Rot/Sal, or PKCδ KO with MA/Sal (one-way ANOVA followed by Fisher's LSD pairwise comparisons).

Fig. 12.

A schematic depiction of the interactive modulation between PKCδ and p47*phox* in MA-induced dopaminergic neurotoxicity. Treatment with a toxic dose of MA resulted in a significant phosphorylation in PKCδ (15 min post-MA). PKCδ-dependent p47*phox* activation was occurred (30 min post-MA). This activation of p47*phox* led to initial enhancements in PHOX activity and ROS formation. These initial oxidative burdens might lead to significant compensative inductions in Nrf-2-dependent glutathione system (i.e., increase in GCLs mRNA, GSH level and glutathione-IR in the neurons and microglia; 30 min post-MA). Thereafter, the initial membrane translocation of p47*phox* (1 h post-MA) was noted, followed by initial dopaminergic losses (2 h post-MA), indicating that initial neurotoxic signals precede the inactivation of Nrf2-dependent system. Importantly, p-p47*phox*-IR in the microglia remains elevated until 1 d post-MA. Further, compensative induction of Nrf-2 level was reduced 1 d post-MA. At that time, GCLs mRNA expression,

GSH level and glutathione (GSH and GSSG)-IR were decreased, while GSSG level was increased and glutathione-IR in the microglial cells remains elevated, and consistently, MA facilitated microglial activity (e.g., increases in Iba-1 labeled immunoreactivity and expression with microglial activation; Supplementary Fig. S4 and S5). Glutathione-IR in the microglia may reflect the necessity for self-protection against oxidative burdens produced by microglia. Consistently, buthionine-sulfoximine (BSO), an inhibitor of glutathione biosynthesis, potentiated microglial activation and pro-apoptotic process (i.e., decreases in p-Akt, p-Bad, p-Bad/14-3-3, Bcl-2, Bcl-xL, whereas increases in TUNEL-positive populations, Bax, cleaved PKC δ and cleaved caspase-3), and led to significant dopaminergic losses. These signaling processes were attenuated by rottlerin (a PKC δ inhibitor), genetic inhibition of PKC δ or p47 $phox$. Rottlerin did not exhibit any additional positive effects against the protective activity offered by genetic inhibition of p47 $phox$. Therefore, we suggest that PKC δ is an important regulator of p47 $phox$ phosphorylation and translocation in acute neurotoxicity induced by MA, and that Nrf-2-related GSH modulation via inhibitions of PKC δ and p47 $phox$ is critical for the dopaminergic protection.