Published in final edited form as:

Biochem Biophys Res Commun. 2013 August 2; 437(3): 380–385. doi:10.1016/j.bbrc.2013.06.085.

PKC δ mediates paraquat-induced Nox1 expression in dopaminergic neurons

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Abstract

Our previous works have shown that the (NADPH) oxidase (Nox) enzyme, in particular Nox1, plays an important role in oxidative stress and subsequent dopaminergic cell death elicited by paraquat (PQ). In non-neuronal and glial cells, protein kinase C δ (PKC δ) shows the ability to regulate the activity of the Nox system. Herein we aimed to investigate if also in dopaminergic neurons exposed to PQ, PKC δ can regulate Nox1 expression.

The chemical inhibitor, rottlerin, and short interference RNA (siRNA) were used to inhibit or selectively knockdown PKC δ , respectively. The studies were performed using the immortalized rat mesencephalic dopaminergic cell line (N27 cells) exposed to PQ, after pre-incubation with rottlerin or transfected with PKC δ -siRNA. We observed that inhibition or knockdown of PKC δ significantly reduced PQ induced Nox1 transcript and protein levels, ROS generation and subsequent dopaminergic cell death. The results suggest that PKC δ plays a role in the regulation of Nox1-mediated oxidative stress elicited by PQ and could have a role in the pathogenesis of Parkinson's disease.

Keywords

NADPH oxidase; Paraquat; Protein Kinase C delta; Parkinson Disease

Introduction

In the central nervous system (CNS), oxidative stress is a major contributor to a number of diseases and aging process, and mitochondria have been considered as the main source of ROS. However, increasing evidence suggests that Nox enzymes might also play a role in ROS production in the CNS [1,2]. Our previous studies have demonstrated that Nox1 has a role in PQ-mediated dopaminergic neuronal cell death both in cell cultures and animal models of Parkinson's disease (PD) [3,4]. Recently, we reported that the activation of Nox1/Rac1 is involved in oxidative stress and consequential dopaminergic neuronal death following 6-hydroxydopamine (6OHDA) treatment [5].

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A growing body of evidence has demonstrated that oxidative damage plays an important role in the pathogenesis of Parkinson Disease (PD). Markers of oxidative stress, such as increased levels of malondialdehyde and cholesterol lipid hydroperoxides, resulting in lipid peroxidation [6,7], have been detected in the substantia nigra (SN) of PD brains. The presence of protein carbonyls and increased level of 8-hydroxy-2-deoxyguanosine, a marker of DNA oxidative damage caused by oxidative stress were also observed in PD postmortem brain tissue [8,9,10].

Epidemiological studies have identified PQ as a risk factors for PD [11], which has been used in laboratory as a neurotoxin to reproduce key pathologic hallmarks of PD [3,12,13]. The mechanism by which PQ induces dopaminergic neuronal toxicity is still under investigation. Previous studies have shown that PQ reduction and consequent superoxide formation are markedly promoted by microglial Nox enzymes [12,14,15]. PQ strongly reduced glial cell viability and its toxic effect was attenuated by a PKC δ inhibitor (rottlerin), an antioxidant (α -tocopherol), and a Nox inhibitor (DPI). These results suggest that PKC δ and ROS play an important role in PQ-induced glial toxicity. In a previous study we have shown that Nox1 mediates oxidative stress and cell death caused by PQ in N27 dopaminergic cells and in mice. We identified for the first time that the Nox1 isoform is constitutively expressed in dopaminergic cells and its level is elevated by PQ administration both *in vivo* and *in vitro* [3]. Moreover, we recently unveil that Nox1-mediated ROS-generation is implicated in alpha-synucleinopathy and dopaminergic cell death induced by PQ [4].

Increasing evidence suggests that transcriptional regulation may be particularly important in the control of Nox1-mediated ROS generation. In fact, Nox1 transcription is induced under various circumstances, such as platelet-derived growth factor, and angiotensin II and prostaglandin F2 α [16,17,18]. In non-neuronal cells such as smooth muscle cells, it was shown that PKC δ is able to regulate Nox1 activity by upregulation of its transcription [19]. It was also reported that PQ toxicity on microglia cells involves increasing levels of ROS through Nox system, which is mediated by PKC δ [20]. A study on phagocytic cells reported that PKC δ is involved in the phosphorylation of p47^{phox} and p67^{phox}, cytosolic components of Nox activation, suggesting that PKC δ is a key mediator of the NADPH enzymes activity. In phagocytic cells, ROS produced by PKC δ -mediated Nox activation causes cell death [21,22]. PKC δ and the Nox system were implicated in the advanced glycation end product (AGE)-induced neuronal toxicity [23]. It has been also demonstrated that the activation of PKC δ and Nox are crucial for the differentiation of neuroblastoma cells induced by retinoic acid [24]. Additionally, PKC δ was linked to dopaminergic cell death, since rottlerin, a PKC δ inhibitor, exerts a neuroprotective effect against MPTP exposure [25].

In the present study, we sought to investigate whether PKC δ could be a regulator of Nox1-mediated oxidative stress and subsequent dopaminergic cell death induced by PQ.

Materials and Methods

Materials

Fetal bovine serum (FBS), RPMI 1640, trypsin/EDTA and penicillin–streptomycin, were purchased from GibcoBRL. Phenylmethylsulfonyl fluoride (PMSF) and Nonidet P-40 (NP-40) were purchased from Sigma Chemicals. Rabbit anti-Nox1 antibody was obtained from Santa Cruz biotechnology (Santa Cruz, CA, USA). Taq polymerase was purchased from Fermentas (Glen Burnie, MD, USA). ECF Western Blotting Reagent Packs kit and anti-rabbit or anti-mouse alkaline phosphatase-linked secondary antibodies were obtained from Amersham Bioscience (Piscataway, NJ, USA). Trizol reagent, 2',7'-Dichlorodihydrofluorescein Diacetate (DCFDA), dihydroethidium (DHE), Lipofectamin

TM, superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). Paraquat (PQ), 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, MO, USA). CytoTox-96-NonRadioactive-Cytotoxicity-Assay for LDH activity was from Promega bioscience (San Luis Obispo, CA; USA). All other chemicals of reagent grade were from Sigma Chemicals or Merck (Rahway, NJ, USA).

Cell-culture

The immortalized rat mesencephalic dopaminergic cell line (N27 cells) was grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 µg/ml), and maintained at 37°C in a humidified atmosphere of 5 % CO₂. Cells were plated on polystyrene tissue-culture plates at a density of 1×10^4 cells/well in 96-well culture plates, 1.5×10^5 cells/well in 6-well culture plates. After 18 hrs, cells were treated with different concentrations of PQ for the indicated duration. For siRNA transfection experiments, cells were plated at a density of 2×10^4 cells/well in 96 well culture plates and of 5×10^5 cells when plated on 60 mm dishes.

Cell Transfection with siRNA

The oligonucleotides targeting to the rat PKC δ mRNA sequence were synthesized chemically, modified into stealth siRNA and purified by Invitrogen. One non-specific siRNA (siRNA-NS) with a similar GC content as PKC δ stealth siRNA was used as negative control. N27 cells were transfected with 56 nM of PKC δ siRNA #1, #2 and #3 (Fig. 2A), at 40–50 % of confluence. Transfection of siRNAs was performed using Lipofectamine 2000 according to the manufacturer's protocol.

Paraquat Treatment

Before PQ (500µM, 800µM and 1000µM) exposure for 6 or 18 hrs, N27 cells were either transfected with PKC δ siRNA for 36 hrs or treated with rottlerin for 3 hrs. The mRNA and protein expression levels of PKC δ and Nox1 were analyzed.

Total RNA Extraction and RT-PCR analysis

Total RNA was extracted from N27 cells using Trizol reagent. Reverse transcription (RT) was performed for 40 min at 42°C with 1 µg of total RNA using 1 unit/µL of superscript II reverse transcriptase. Random primers were used as primers. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA (1 µl) obtained from 1 µg of total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on Genbank entries for: rat Nox1 (sense: 5'-TGACAGTGATGTATGCAGCAT-3', antisense: 5'-CAGCTTGTGTGTGCACGCTG-3'); rat PKC δ (sense: 5'-AGCCTCTCCCTCTCTCCAC-3', antisense: 5'-GGTGGGCTTCTTCTGTACCA3'); rat GAPDH (sense: 5'-ATCACCATCTTCCAGGAGCG-3', antisense: 5'-GATGGCATGGACTGTGGTCA-3'). PCR reaction solutions contained 10 µl of 2× PCR buffer, 1.25 mM of each dNTP, 1 pmol each of forward and reverse primers, and 2.5 units of Taq polymerase to a final volume of 20 µl. PKC δ , amplification was achieved in 35 cycles of 40 sec at 95°C, 40 sec at 52°C, and 40 sec at 72°C. Nox1, amplification was achieved using 38 cycles of 40 sec at 95°C, 30 sec at 62°C, and 2 min at 72°C. After the last cycle, all samples were incubated for additional 7 min at 72°C. PCR fragments were analyzed on a 1% agarose gel containing ethidium bromide. Results were quantified using Quantity One Software (Bio-Rad). All values were normalized against the amplified GAPDH. Each primer set specifically recognized only the gene of interest as indicated by amplification of a single band of the expected size.

Western-blot analysis

Cells were lysed on ice with cold RIPA buffer to collect protein. Equal amounts of protein were loaded in each lane of a 12.5 % SDS polyacrylamide gel. After electrophoresis and transfer onto a polyvinylidene difluoride (PVDF) membrane, specific protein bands were detected using the primary antibodies rabbit anti-Nox1, rabbit anti-PKC δ or mouse anti- β -actin and secondary antibodies anti-rabbit or anti-mouse IgG, followed by enhanced chemifluorescence system detection. All values were normalized against β -actin levels.

Determination of cellular ROS content

ROS levels were measured by DCFDA, DHE and NBT assay. After PQ treatment, cells were incubated with DCFDA (100 μ M), DHE (100 μ M) and NBT (0.3 mg/ml) in complete medium for 1, 4 and 6 hrs at 37°C, respectively. To measure the fluorescence produced in the DCFDA and DHE, the emitted fluorescence was read in a microplate spectrophotometer plate reader using Ex/Em 485/535 nm and 590/620 nm for DCFDA and DHE, respectively. To quantify NBT precipitation, cells were washed twice with 70 % methanol and fixed for 5 min in 100 % methanol. Wells were allowed to air dry and the water insoluble formazan was solubilized with 120 μ l 2 M KOH and 140 μ l DMSO. The optical density was read in a microplate spectrophotometer plate reader at 590 nm.

Cell viability assays

To assess cell viability, the levels of MTT reduction and LDH were measured. 0.5 mg/ml of MTT solution was added to cells and incubated at 37°C for 90 min. The formazan precipitates were solubilized with acidic isopropanol (0.04M HCl in absolute isopropanol). The absorbance of the solubilized formazan crystals was measured at a wavelength of 570 nm using a microplate reader (BioRad). LDH activity in the cell-free extracellular supernatant was quantified as an indicator of cell death, using the cytotoxic assay kit as indicated by the manufacturer.

Data analysis and statistics

Statistical analysis was carried out with GraphPad Prism v.5 (GraphPad Software Inc., San Diego, CA). Data are expressed as percentages of control conditions, and are presented as mean $\pm$ SEM of at least three experiments, performed in triplicate, in independent cell cultures. Statistical analyses were performed using one-way ANOVA followed by Dunnett's test or Bonferroni's Multiple Comparison Test as indicated in figure legends. Values of $p < 0.05$ were considered significant.

Results

Attenuation of PQ-mediated Nox1 expression, ROS generation and dopaminergic cell death by the inhibition of PKC δ

To clarify the potential involvement of PKC δ in the expression of Nox1, we have evaluated the mRNA levels of Nox1 in N27 dopaminergic cells exposed to 500 μ M of PQ for 6 hrs, in the presence or absence of PKC δ inhibitor rottlerin. As shown in Fig. 1A, increased Nox1 mRNA induced by PQ was reversed in cells pre-treated with 5 μ M of rottlerin for 3 hrs, suggesting that PKC δ may be a potential mediator of the Nox1 induction by PQ.

We have further investigated the role of PKC δ in PQ-mediated ROS generation and dopaminergic cells death. Cells were pre-treated with 5 μ M of rottlerin for 3 hrs prior to the exposure with different concentrations of PQ (100, 500, 800 or 1000 μ M). ROS levels were measured 24 hrs after PQ treatment. In cells treated with 100, 500, 800 or 1000 μ M of PQ, ROS levels were significantly increased by 53%; 62%; 73% and 110%, respectively, as

compared with control cells. The inhibition of PKC δ with rottlerin significantly reduced PQ-mediated ROS generation (Fig. 1B). The effect of rottlerin on PQ-mediated dopaminergic cell death was also tested. The viability of cells was determined by MTT assays (Fig. 1C). The levels of reduced MTT were statistically higher in cultures pre-treated with rottlerin as compared to ones treated with PQ only. As shown in Fig. 1C, rottlerin increased the levels of reduced MTT by 25% and 24% in cultures treated with 500 and 800 μ M of PQ, respectively, as compared with cultures exposed to PQ only.

These results suggest that PKC δ can be a central mediator of ROS generation and dopaminergic cell death induced by PQ exposure.

Selective knockdown of PKC δ expression by siRNA

Although rottlerin has been widely used as a specific PKC δ inhibitor, recent studies suggest that it may affect other non-specific targets [26], leading us to apply a knockdown strategy using PKC δ siRNA to better understand the role of PKC δ in PQ neurotoxicity. Three different siRNA sequences against PKC δ were tested in N27 cells and a non-specific siRNA was used as a negative control. Knockdown efficiency was evaluated by RT-PCR and immunoblot analysis. The RT-PCR analyses revealed that even though all siRNA sequences reduce PKC δ mRNA levels, only siPKC δ #3 significantly reduce PKC δ mRNA levels (Fig. 2B). The siPKC δ #1 reduced PKC δ mRNA level by 18% and siPKC δ #2 did by 22%. The most effective siRNA sequence was siPKC δ #3, as significantly reducing PKC δ mRNA levels by 32%, as compared to non-specific siRNA transfected cells.

PKC δ protein levels were also decreased accordingly by 29%, 37%, and 55% after transfection with PKC δ siRNA #1, siRNA #2 and siRNA #3, respectively, as compared with non-specific siRNA transfected cells (Fig. 2C). Based on this, PKC δ siRNA #3 was selected to be used in further experiments.

PKC δ gene silencing decreases PQ-induced Nox1 expression

In order to understand if PKC δ may regulate the Nox1 expression induced by PQ, we investigated whether PKC δ knockdown influence Nox1 expression in dopaminergic cells exposed to PQ.

N27 cells were transfected with PKC δ siRNA #3 36 hrs prior to exposure to different concentrations of PQ (500, 800 or 1000 μ M). Nox1 mRNA and protein levels were determined by RT-PCR and Western blot, respectively.

Nox1 mRNA levels were significantly increased in N27 cells treated with PQ (Fig. 3A), and PKC δ knockdown significantly reduced this increases by 34% and 40% for 800 μ M and 1000 μ M of PQ, respectively as compared with cells exposed to PQ alone. PQ-induced Nox1 protein expression was also decreased by PKC δ knockdown. As shown in Fig 3B, PKC δ knockdown significantly reduced Nox1 protein levels by 21% and 44%, which were induced by 800 μ M and 1000 μ M of PQ, respectively, as compared with cells exposed to PQ only.

These results suggest that PKC δ can play an important role in the PQ-mediated up-regulation of Nox1 in dopaminergic cells.

PKC δ knockdown reduces ROS and cell death induced by PQ

To investigate the role of PKC δ in PQ-induced ROS generation, N27 cells were transfected with PKC δ siRNA and treated with 500 μ M, 800 μ M and 1000 μ M of PQ. ROS levels were measured 24 hrs after PQ treatment using DCFDA and DHE assays. The analysis with

DCFDA assay (Fig. 4A) revealed that ROS levels of 127% observed in cells treated with 1000 μ M PQ were significantly decreased to 96% after PKC δ knockdown. Similar results were observed using the DHE assay (Fig. 4B). Significant decrease in superoxide levels was also observed. 149% and 162% of superoxide in cells treated with 800 μ M and 1000 μ M of PQ, respectively, were reduced to 116% and 117% after PKC δ knockdown. To evaluate the effect of PQ on dopaminergic cell death after PKC δ knockdown, we measured cell survival and death using MTT reduction and LDH release assay, respectively in PKC δ knock-down N27 cells after treatment with PQ (500 μ M, 800 μ M and 1000 μ M) for 24 hrs.

As shown in Fig. 4C, as compared with non-transfected cells, significant decreases in MTT reduction were observed by different concentrations of PQ (67%, 53% and 39% for 500 μ M, 800 μ M or 1000 μ M of PQ, respectively), which was reversed by PKC δ knockdown (99%, 96% and 91% for 500 μ M, 800 μ M and 1000 μ M of PQ, respectively). Similar results were obtained with the LDH-based cell death assay that measures LDH released to the medium (Fig. 4D). Compared with non-transfected cells, the LDH levels of N27 cells treated with 500 μ M, 800 μ M and 1000 μ M of PQ were significantly increased to 132%, 140% and 153%, respectively. PKC δ knockdown significantly reduced LDH release induced by 500 μ M, 800 μ M and 1000 μ M of PQ, to 107%, 98% and 114%, respectively.

These results suggest that PKC δ is a mediator in PQ-mediated ROS generation and subsequent dopaminergic cell death.

Discussion

In the current study, we showed that PKC δ could be a key mediator of Nox1 expression in dopaminergic cells after exposure to PQ. Using either a chemical inhibitor, rottlerin, or RNAi-mediated PKC δ knockdown strategy, PQ-mediated Nox1 expression, ROS generation and consequent dopaminergic cell death were significantly attenuated.

Dopaminergic neurons are known to be highly susceptible to oxidative stress insults because of their reduced antioxidant capability, high content of dopamine, melanin and lipids, which renders dopaminergic neurons prone to oxidative damage [27]. PQ is a widely used herbicide, shown to cause oxidative stress and the selective death of dopaminergic neurons, reproducing the primary neurodegenerative feature of PD [28]. ROS have important biological functions and Nox can generate ROS in a regulated manner in several circumstances [1]. In our previous studies, we showed that dopaminergic neurons were equipped with the Nox system and that particularly Nox1 is a key player in PQ-induced toxicity to dopaminergic neurons [3,4]. PKC δ is a member of the Protein kinase C (PKC) family and is generally considered a growth inhibitor or a pro-apoptotic PKC [29,30]. However, the pathway in which PKC δ leads to apoptosis remains unclear. Miller and collaborators [20] showed that PQ toxicity on microglia cells involves Nox-mediated ROS increase which is regulated by PKC δ . In human neurons, it was reported that PKC δ can increase DNA binding activity of redox-sensitive transcription factor AP-1 [31] which is known to be involved in the regulation of Nox enzymes [32,33]. This could be suggestive of a mechanism by which PKC δ regulates Nox1 transcriptional regulation. Another report showed that PKC δ mediates PQ-induced ROS generation and consequential death of glial cells [34]. PQ also activates signaling pathways such as PKC δ or MAPK, which are known to be Nox1 transcriptional activators [19,32]. In these studies it was demonstrated that in non-neuronal cells, like smooth muscle cells, PKC δ regulates Nox activity by upregulation of Nox1 at its mRNA level. In accordance with these works, the current work showed that PKC δ plays a key role in Nox1 upregulation, ROS generation and subsequent dopaminergic cell death induced by PQ. Either rottlerin, or PKC δ knockdown successfully reversed aforementioned PQ effects on dopaminergic cells. The role of PKC δ in dopaminergic

neurons has been reported in another toxin-based PD model in which rottlerin exerts neuroprotective effects on dopaminergic neurons after exposure to MPTP, a well-known dopaminergic neurotoxin [25].

In summary, our data demonstrate that PKC δ is an important mediator of PQ-induced ROS production through Nox1 complex in dopaminergic neurons. It is likely that PQ sequentially induces PKC δ activation, Nox1 expression, ROS generation and finally leads to dopaminergic cell death, suggesting that this pathway could serve as a valuable therapeutic target for the treatment PD.

Acknowledgments

This work was supported by the US National Institute of Health [grant number: RO1 NS062827 to Y.S.K.] and Michael J. Fox Foundation [grant number: Target Validation 2009 to Y.S.K.].

Ana Clara Cristóvão supported by the Portuguese Foundation for Science and Technology – FCT (SFRH/BPD/69643/2010).

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Highlights

Paraquat (PQ)-mediated Nox1 expression is mediated by PKC δ .

Rottlerin reduces PQ-mediated Nox1 expression, ROS levels and dopaminergic cell loss.

PKC δ knockdown decreases Nox1 expression, ROS levels and cell death induced by PQ.

PKC δ could serve as a therapeutic target for new treatments for Parkinson Disease.

Fig. 1.

Decreases of PQ-mediated Nox1 expression, ROS levels and N27 dopaminergic cell death by a PKC δ inhibitor, rottlerin. **(A)** Nox1 mRNA expression levels, **(B)** ROS levels measured using NBT assay, and **(C)** cell death levels assessed by MTT reduction assay, in cells treated with rottlerin (R) and exposed to PQ. The results are expressed as a percentage of their controls. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control cultures. + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ vs. culture condition with PQ treatment and without rottlerin pre-treatment.

Fig. 2.

Specific siRNAs-mediated knockdown of PKC δ in N27 cells. **(A)** Three PKC δ -siRNA (siPKC δ) sequences tested. **(B)** PKC δ mRNA expression levels and **(C)** PKC δ protein expression levels in cells transfected with siPKC δ #1, siPKC δ #2, siPKC δ #3 and siRNA-NS and in non-transfected cells (Non-tx). Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs. Non-Tx cells.

Fig. 3.

Reduction of PQ-induced Nox1 expression by PKC δ knockdown. **(A)** Nox1 mRNA expression levels of cells transfected with PKC δ siRNA and siRNA-NS followed by PQ treatment. **(B)** Nox1 protein expression levels of cells transfected with PKC δ -siRNA and siRNA-NS. Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison Test and Bonferroni's Multiple Comparison Test. * $P < 0,05$; ** $P < 0,01$ and *** $P < 0,001$ vs. Non-tx cells. + $P < 0,05$; ++ $P < 0,01$; +++ $P < 0,001$ vs. culture condition of each PQ treatment without transfection.

Fig. 4.

PKC δ gene silencing reduces ROS and cell death induced by PQ. ROS levels in N27 cells transfected with PKC δ siRNA followed by PQ treatments measured using (A) DCFDA or (B) DHE. (C) Cell viability analysis using MTT assay. (D) Cell death levels quantified by LDH assay. Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison Test and Bonferroni's Multiple Comparison Test. * $p < 0,05$; ** $p < 0,01$ and *** $p < 0,001$ vs. Non-Tx cells. + $p < 0,05$; ++ $p < 0,01$; +++ $p < 0,001$ vs. culture condition of each PQ treatment without transfection.