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PKCδ 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 (PKCδ) shows the ability to regulate the activity of the Nox system. Herein we aimed to investigate if also in dopaminergic neurons exposed to PQ, PKCδ can regulate Nox1 expression.

The chemical inhibitor, rottlerin, and short interference RNA (siRNA) were used to inhibit or selectively knockdown PKCδ, 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 PKCδ-siRNA. We observed that inhibition or knockdown of PKCδ significantly reduced PQ induced Nox1 transcript and protein levels, ROS generation and subsequent dopaminergic cell death. The results suggest that PKCδ 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.

1. 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].

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 [8–10].

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 PKCδ inhibitor (rottlerin), an antioxidant (α-tocopherol), and a Nox inhibitor (DPI). These results suggest that PKCδ 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 F2α [16–18]. In non-neuronal cells such as smooth muscle cells, it was shown that PKCδ is able to regulate Nox1 activity by upregulation of its transcription [19]. It was also reported that PQ toxicity on microglia cells in-

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volves increasing levels of ROS through Nox system, which is mediated by PKCδ [20]. A study on phagocytic cells reported that PKCδ is involved in the phosphorylation of $p47^{phox}$ and $p67^{phox}$, cytosolic components of Nox activation, suggesting that PKCδ is a key mediator of the NADPH enzymes activity. In phagocytic cells, ROS produced by PKCδ-mediated Nox activation causes cell death [21,22]. PKCδ 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 PKCδ and Nox are crucial for the differentiation of neuroblastoma cells induced by retinoic acid [24]. Additionally, PKCδ was linked to dopaminergic cell death, since rottlerin, a PKCδ inhibitor, exerts a neuroprotective effect against MPTP exposure [25].

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

2. Materials and methods

2.1. 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).

2.2. 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% CO_2. Cells were plated on polystyrene tissue-culture plates at a density of 1×10^4 cells/well in 96-well culture plates, 1.5 x 10^5 cells/well in 6-well culture plates. After 18 h, 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.

2.3. Cell transfection with siRNA

The oligonucleotides targeting to the rat PKCδ 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 PKCδ stealth siRNA was used as negative control. N27 cells were transfected with 56 nM of PKCδ 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.

2.4. Paraquat treatment

Before PQ (500, 800 and 1000 μM) exposure for 6 or 18 h, N27 cells were either transfected with PKCδ siRNA for 36 h or treated with rottlerin for 3 h. The mRNA and protein expression levels of PKCδ and Nox1were analyzed.

2.5. 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 Genebank entries for: rat Nox1 (sense: 5'-TGACAGTGATGTATGCAGCAT-3', antisense: 5'-CAGCTTGTTGTGTGCACGCTG-3'); rat PKCδ (sense: 5'-AGCCTCTCCCTCTCTTCCAC-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. PKCδ, amplification was achieved in 35 cycles

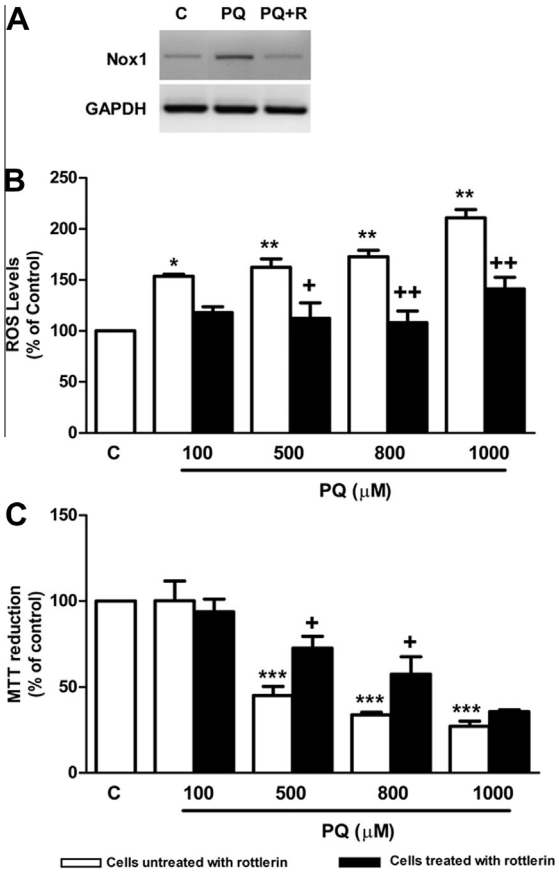

Fig. 1. Decreases of PQ-mediated Nox1 expression, ROS levels and N27 dopaminergic cell death by a PKCδ inhibitor, rottlerin. (A) Nox1 mRNA expression levels, (B) ROS levels measured using NBT assay, and (C) cell death levels assessed by MTT reduction assay, in cells treated with rottlerin (R) and exposed 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.

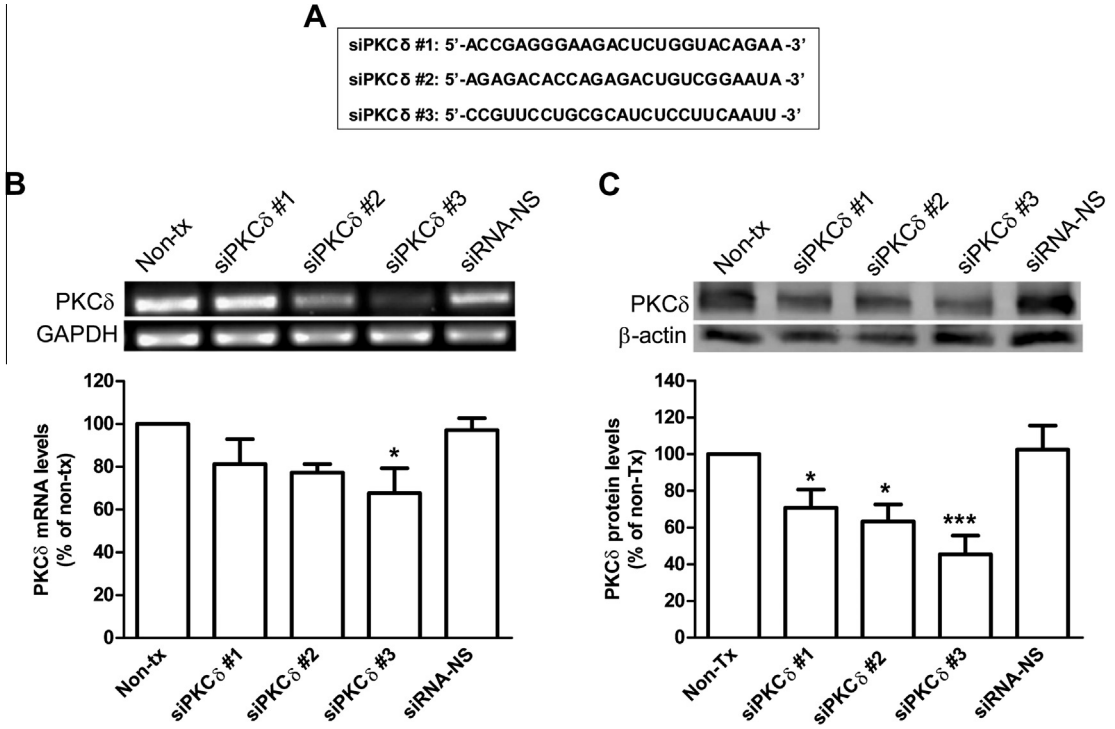

Fig. 2. Specific siRNAs-mediated knockdown of PKCδ in N27 cells. (A) Three PKCδ-siRNA (siPKCδ) sequences tested. (B) PKCδ mRNA expression levels and (C) PKCδ protein expression levels in cells transfected with siPKCδ #1, siPKCδ #2, siPKCδ #3 and siRNA-NS and in non-transfected cells (Non-tx). Statistical 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.

of 40 s at 95 °C, 40 s at 52 °C, and 40 s at 72 °C. Nox1, amplification was achieved using 38 cycles of 40 s at 95 °C, 30 s 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.

2.6. 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-PKCδ 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.

2.7. Determination of cellular ROS content

ROS levels were measured by DCFDA, DHE and NBT assay. After PQ treatment, cells were incubated with DCFDA (100 μM), DHE (100 μM) and NBT (0.3 mg/ml) in complete medium for 1, 4 and 6 h 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 μl 2 M KOH and 140 μl DMSO. The optical density was read in a microplate spectrophotometer plate reader at 590 nm.

2.8. 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 (0,04 M HCl in absolute ispropanol). 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.

2.9. 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 ± 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.

3. Results

3.1. Attenuation of PQ-mediated Nox1 expression, ROS generation and dopaminergic cell death by the inhibition of PKCδ

To clarify the potential involvement of PKCδ in the expression of Nox1, we have evaluated the mRNA levels of Nox1 in N27 dopaminergic cells exposed to 500 μM of PQ for 6 h, in the presence or ab-

sence of PKCδ inhibitor rottlerin. As shown in Fig. 1(A), increased Nox1 mRNA induced by PQ was reversed in cells pre-treated with 5 μM of rottlerin for 3 h, suggesting that PKCδ may be a potential mediator of the Nox1 induction by PQ.

We have further investigated the role of PKCδ in PQ-mediated ROS generation and dopaminergic cells death. Cells were pre-treated with 5 μM of rottlerin for 3 h prior to the exposure with different concentrations of PQ (100, 500, 800 or 1000 μM). ROS levels were measured 24 h after PQ treatment. In cells treated with 100, 500, 800 or 1000 μM of PQ, ROS levels were significantly increased by 53%; 62%; 73% and 110%, respectively, as compared with control cells. The inhibition of PKCδ 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 μM of PQ, respectively, as compared with cultures exposed to PQ only.

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

3.2. Selective knockdown of PKCδ expression by siRNA

Although rottlerin has been widely used as a specific PKCδ inhibitor, recent studies suggest that it may affect other non-specific targets [26], leading us to apply a knockdown strategy using PKCδ siRNA to better understand the role of PKCδ in PQ neurotoxicity. Three different siRNA sequences against PKCδ 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 PKCδ mRNA levels, only siPKCδ #3 significantly reduce PKCδ mRNA levels (Fig. 2B). The siPKCδ #1 reduced PKCδ mRNA level by 18% and siPKCδ#2 did by 22%. The most effective siRNA sequence was siPKCδ #3, as significantly reducing PKCδ mRNA levels by 32%, as compared to non-specific siRNA transfected cells.

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

3.3. PKCδ gene silencing decreases PQ-induced Nox1 expression

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

N27 cells were transfected with PKCδ siRNA #3 36 h prior to exposure to different concentrations of PQ (500, 800 or 1000 μM). 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 PKCδ knockdown significantly reduced this increases by 34% and 40% for 800 and 1000 μM of PQ, respectively as compared with cells exposed to PQalone. PQ-induced Nox1 protein expression was also decreased by PKCδ knockdown. As shown in Fig 3B, PKCδ knockdown significantly reduced Nox1 protein levels by 21% and 44%, which were induced by 800 and 1000 μM of PQ, respectively, as compared with cells exposed to PQ only.

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

3.4. PKCδ knockdown reduces ROS and cell death induced by PQ

To investigate the role of PKCδ in PQ-induced ROS generation, N27 cells were transfected with PKCδ siRNA and treated with 500, 800 and 1000 μM of PQ. ROS levels were measured 24 h after PQ treatment using DCFDA and DHE assays.

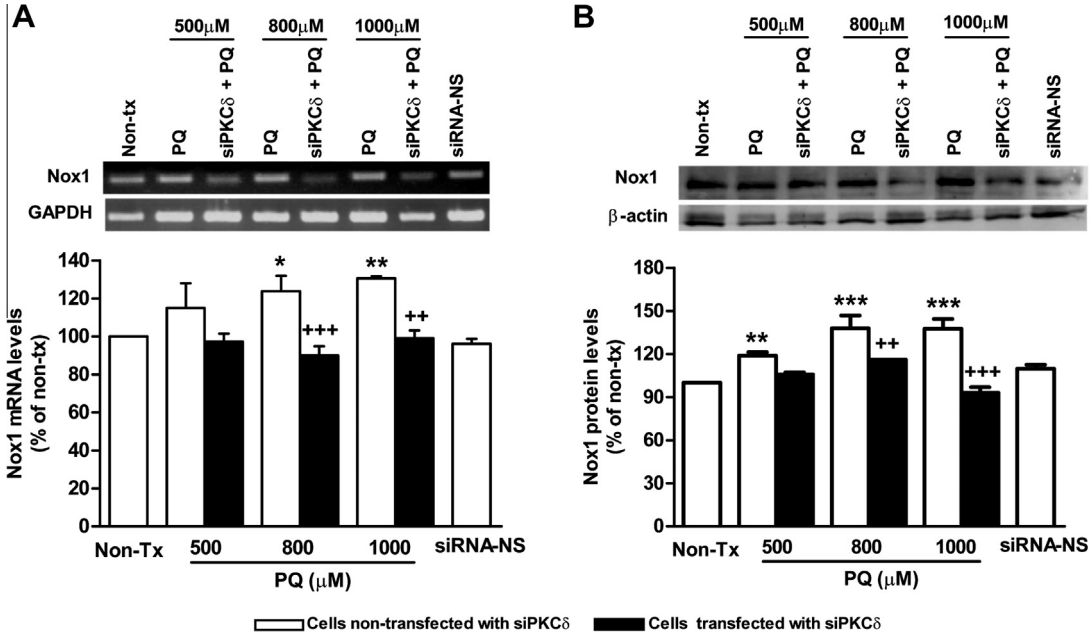

Fig. 3. Reduction of PQ-induced Nox1 expression by PKCδ knockdown. (A) Nox1 mRNA expression levels of cells transfected with PKCδ siRNA and siRNA-NS followed by PQ treatment. (B) Nox1 protein expression levels of cells transfected with PKCδ-siRNA and siRNA-NS. Statistical analysis was performed using 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.

The analysis with DCFDA assay (Fig. 4A) revealed that ROS levels of 127% observed in cells treated with 1000 μM PQ were significantly decreased to 96% after PKCδ 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 and 1000 μM of PQ, respectively, were reduced to 116% and 117% after PKCδ knockdown. To evaluate the effect of PQ on dopaminergic cell death after PKCδ knockdown, we measured cell survival and death using MTT reduction and LDH release assay, respectively in PKCδ knock-down N27 cells after treatment with PQ (500, 800 and 1000 μM) for 24 h.

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, 800 or 1000 μM of PQ, respectively), which was reversed by PKCδ knockdown (99%, 96% and 91% for 500, 800 and 1000 μM 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-transfectedcells, the LDH levels of N27 cells treated with 500, 800 and 1000 μM of PQ were significantly increased to 132%, 140% and 153%, respectively. PKCδ knockdown significantly reduced LDH release induced by 500, 800 and 1000 μM of PQ, to 107%, 98% and 114%, respectively.

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

4. Discussion

In the current study, we showed that PKCδ could be a key mediator of Nox1 expression in dopaminergic cells after exposure to PQ. Using either a chemical inhibitor, rottlerin, or RNAi-mediated PKCδ 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]. PKCδ 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 PKCδ 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 PKCδ. In human neurons, it was reported that PKCδ 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 PKCδ regulates Nox1 transcriptional regulation. Another report showed that PKCδ mediates PQ-induced ROS generation and consequential death of glial cells [34]. PQ also activates signaling pathways such as PKCδ 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, PKCδ regulates Nox activity by upregulation of Nox1 at its mRNA level. In accordance with these works, the current work showed that PKCδ plays a key role in Nox1 upregulation, ROS generation and subsequent dopaminergic cell death induced by PQ. Either rottlerin, or PKCδ knockdown successfully reversed

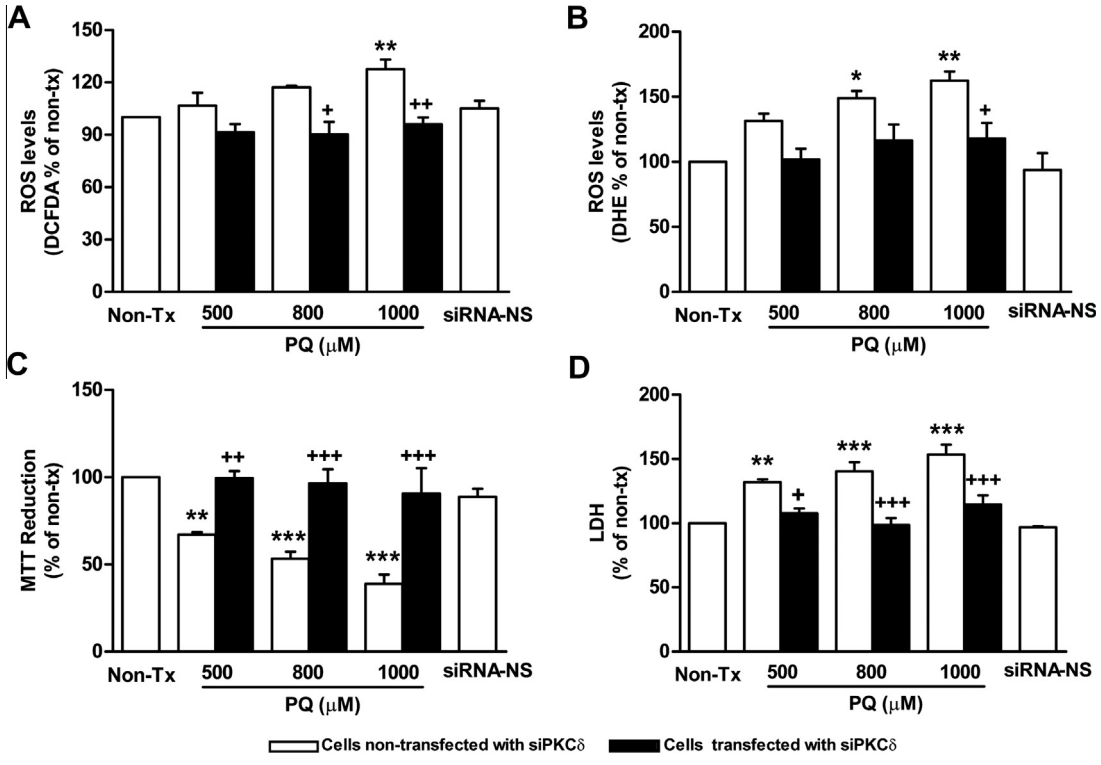

Fig. 4. PKCδ gene silencing reduces ROS and cell death induced by PQ. ROS levels in N27 cells transfected with PKCδ siRNA followed by PQ treatments measured using (A) DCFDA or (B) DHE. (C) Cell viability analysis using MTT assay. (D) Cell death levels quantified by LDH assay. Statistical analysis was 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.

aforementioned PQ effects on dopaminergic cells. The role of PKCδ 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 PKCδ is an important mediator of PQ-induced ROS production through Nox1 complex in dopaminergic neurons. It is likely that PQ sequentially induces PKCδ 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

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