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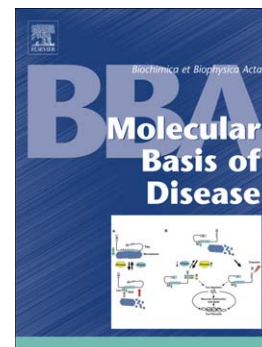
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**Activation of PKC δ and ERK1/2 in the Sensitivity to UV-Induced Apoptosis of
Human Cells Harboring 4977 bp Deletion of Mitochondrial DNA**

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Summary

The 4977 bp deletion of mitochondrial DNA (mtDNA), often found in patients with chronic progressive external ophthalmoplegia (CPEO), has been demonstrated to increase the susceptibility to apoptosis of human cells. We investigated the mechanism underlying the apoptotic susceptibility of the Δ 4977 cybrid harboring about 80% 4977 bp-deleted mtDNA. The production of hydrogen peroxide (H_2O_2) and phosphorylation of PKC δ and ERK1/2 were increased in the Δ 4977 cybrid, which was more susceptible to UV-induced apoptosis. Moreover, treatment with N-acetyl-L-cysteine (NAC) or blocking of activation of PKC δ by rottlerin or PKC δ -siRNA, and inhibition of ERK1/2 by PD98059 or ERK1/2-siRNA significantly attenuated the susceptibility of the Δ 4977 cybrid to apoptosis. Furthermore, the increase of PKC δ expression in the Δ 4977 cybrid also amplified the apoptotic signal through caspase 3-mediated proteolytic activation of PKC δ . In addition, PKC δ and ERK1/2 were hyperphosphorylated in skin fibroblasts of CPEO patients harboring 4977 bp-deleted mtDNA. We suggest that the activation of PKC δ and ERK1/2 elicited by 4977 bp-deleted mtDNA-induced oxidative stress plays a role in the susceptibility of the mutant cells to apoptosis. This may explain, at least in part, the degenerative manifestation of brain and muscle in patients with mitochondrial encephalomyopathies such as CPEO syndrome.

1. Introduction

Mitochondria are intracellular organelles that are responsible for energy production and are also the major source of reactive oxygen species (ROS) in mammalian cells. More than 100 mutations of mtDNA are associated with mitochondrial diseases and many of them have been proved to cause mitochondrial dysfunction [1]. CPEO syndrome is one of the mitochondrial encephalomyopathies characterized by bilateral ptosis, limitation of extraocular muscle movement, and is often associated with large-scale deletions of mtDNA, including the most common 4977 bp deletion [2]. Previous studies of cybrids harboring 4977 bp-deleted mtDNA showed that mitochondrial respiratory function was decreased and the levels of oxidative damage to DNA and lipids were increased with the proportion of 4977 bp-deleted mtDNA [3]. The intracellular levels of ROS and the 8-hydroxy 2'-deoxyguanosine (8-OHdG) in fibroblasts of CPEO patients were higher than those of controls [4]. Moreover, apoptotic hallmarks of caspase 3 activation, cytochrome *c* release and DNA fragmentation were observed in the affected muscle fibers carrying pathogenic mtDNA mutations [5,6], and in the cybrids harboring mutated mtDNA upon treatment with different apoptotic stimuli [7-11]. In previous studies, we demonstrated that mtDNA mutation increased the susceptibility of human cells to apoptosis induced by UV irradiation or staurosporine [7,8]. Furthermore, coenzyme Q₁₀ was found to attenuate UV-induced apoptosis of human cells harboring mutated mtDNA [12]. These findings

suggest that ROS may play an important role in the susceptibility to apoptosis of human cells harboring a pathogenic mutation of mtDNA. However, the molecular mechanism remains unclear.

PKC δ belongs to the novel forms of the serine/threonine PKC family that are Ca²⁺-independent and diacylglycerol-dependent, and emerges as a pivotal effector of apoptosis triggered by apoptotic stimuli including genotoxins, oxidative stress and UV irradiation [13]. The activation of PKC δ by phosphorylation and caspase 3-mediated proteolytic cleavage not only turns on the MAPKs (mitogen-activated protein kinases) and other signaling pathways to alter the transcription of apoptotic genes, but also enables PKC δ itself to target to mitochondrial proteins (e.g., phospholipid scramblase 3) and nuclear proteins (e.g., DNA-PK, lamin B and Rad 4) to process and amplify apoptotic signals [14,15]. Overexpression of the catalytic fragment and full-length protein of PKC δ could induce apoptotic hallmarks including Bax activation, cytochrome *c* release, chromatin condensation and DNA fragmentation [16-19]. On the other hand, inhibition of PKC δ or overexpression of kinase-inactive and non-cleavable PKC δ mutants could reduce apoptosis triggered by UV irradiation, chemotherapeutic agents or oxidative stress [17,20-22]. Moreover, H₂O₂-elicited threonine and tyrosine phosphorylation of PKC δ increased the kinase activity [23], and triggered the loss of mitochondrial membrane potential and release of cytochrome *c* [15]. In addition, blocking PKC δ signaling could markedly

increase the resistance of cells to H₂O₂-induced apoptosis [17]. In a previous study, we demonstrated that PKC δ is involved in the regulation of mitochondrial biogenesis and intracellular H₂O₂ in human cells, and that overexpression of PKC δ is involved in abnormal proliferation of mitochondria in affected tissues of patients with MERRF (myoclonic epilepsy and ragged-red fibers) syndrome [23]. This scenario may play a role in the pathophysiology and clinical manifestation of mitochondrial diseases.

ERK1/2 is a member of the MAPKs family, and is activated by the phosphorylation of a threonine and a tyrosine residue by the dual-specificity kinases MEK1 and MEK2 in response to various stimuli such as growth factors and oxidative stress. Following activation through the upstream Ras-Raf-MEK signaling pathway, ERK1/2 phosphorylates a variety of substrates including Elk-1, p90RSK and c-Myc to mediate the expression of downstream genes involved in the proliferation, survival and differentiation of the target cells [24]. However, recent studies revealed a detrimental role of ERK1/2 signaling in apoptosis induced by different stimuli [25,26]. ERK1/2 was found to contribute to ROS-induced cell death, and inhibition of ERK1/2 by PD98059 could rescue cells from ROS-triggered apoptosis [27,28]. Moreover, phorbol ester, a PKC activator, was found to cause a rapid and massive activation of ERK1/2 through the Raf-MEK-ERK pathway [29]. These observations suggest that PKC δ may be involved in the upstream signaling of the ERK1/2 pathway.

We report here that the increase of susceptibility to UV-induced apoptosis of human cells harboring 4977 bp-deleted mtDNA is associated with ROS-elicited activation of the PKC δ and ERK1/2 signaling pathways.

2. Materials and Methods

2.1. Chemicals and antibodies

Rottlerin and PD98059 were purchased from Calbiochem Inc. (San Diego, CA); NAC was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). PKC δ and ERK1/2 antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA), and GAPDH antibody was purchased from Millipore (Billerica, MA). The other antibodies were obtained from Cell Signaling Technology (Danvers, MA) unless indicated otherwise.

2.2. Cell culture

The cybrids harboring wild-type and about 80% of mtDNA with 4977 bp deletion (termed the Δ 4977 cybrid) were made by fusing mtDNA-less (ρ^0) human 143B osteosarcoma cells with enucleated skin fibroblasts established from a patient with clinically proven CPEO syndrome, and were characterized by Southern hybridization as previously described [3]. The cybrids were grown in DMEM supplemented with 5% FBS, 100 μ g/ml pyruvate, 50 μ g/ml uridine, 200 units/ml penicillin G, 200 μ g/ml streptomycin sulfate and 0.5 μ g/ml amphotericin B at 37°C in humidified 5% CO₂/95% air. The primary cultures of skin fibroblasts were established from 3 patients with CPEO syndrome and 3 healthy normal subjects, respectively, and were used at passages 8 to 12. The proportions of 4977 bp-deleted mtDNA in skin fibroblasts from the 3 CPEO patients were about 6.5, 6.5 and 4.5%, respectively. Experiments were performed on skin fibroblasts grown to 80%

confluence in 100-mm culture dishes in DMEM supplemented with 5% FBS, 200 units/ml penicillin G, 200 µg/ml streptomycin sulfate and 0.5 µg/ml amphotericin B at 37°C in humidified 5% CO₂/ 95% air.

2.3. *Measurement of oxygen consumption rate*

The oxygen consumption rate of endogenous respiration (non-maximal respiration rate) of the cells was measured by the 782 Oxygen Meter (Strathkelvin Instruments, Scotland, UK) with a water circulation system to maintain the reaction condition at 37°C. After the machine had been stabilized, about 1.0×10^6 cells were collected by trypsinization and suspended in 330 µl assay buffer (125 mM sucrose, 65 mM KCl, 2 mM MgCl₂, 20 mM phosphate buffer, pH 7.2), and then transferred into the incubation chamber. After recording the rate of oxygen consumption of the cybrids for 3 min, 2 µl of 250 mM KCN was added into the chamber to inhibit mitochondrial respiration to record the non-mitochondrial oxygen consumption for another 3 min by the SI 782 Oxygen System software version 3.0. The assay measured the endogenous respiration of the cells, which is not the maximal respiration rate.

2.4. *Measurement of H₂O₂ production*

The production of H₂O₂ by cells was measured by using a highly sensitive and specific fluorescent dye, *N*-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red; Invitrogen,

Billerica, MA) [30]. A total volume of 500 μ l assay mixture containing 50 μ M Amplex Red reagent and 0.1 U/ml HRP in Krebs-Ringer phosphate buffer (KRPB; 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl_2 , 1.22 mM MgSO_4 , 5.5 mM glucose, pH 7.4) was added to the 12-well plate with 7.5×10^4 cells/well. After incubation for 1 h in the dark at 37°C, the reaction mixture was transferred to a 96-well plate, and subjected to fluorescence measurement in a microplate reader (Fluoroskan Ascent, Thermo, MA) at the excitation and emission wavelengths of 538 nm and 590 nm, respectively. In order to normalize the signals, the cell number was determined by the AlamarBlue assay (AbD Serotec, Oxford, UK) according to the manufacturer's protocol.

2.5. Induction of apoptosis

Approximately 8.0×10^5 cells were grown in 100-mm culture dishes for 24 h after seeding, and then exposed to UV irradiation at 40 J/m². After UV irradiation, the cells were quickly added with fresh DMEM and allowed to continue growing for 24 h before assay of the caspase 3 activity. To test the inhibitory effects of NAC, rottlerin and PD98059 on the susceptibility to UV-induced apoptosis, the cells were treated with indicated concentrations of each drug for 18 h before exposure to UV and addition of fresh DMEM containing the indicated drugs after UV irradiation.

2.6. Measurement of caspase 3 activity

Cell lysate was prepared by repeated freeze-thawing of the cybrids in 100 μ l extraction buffer (12.5 mM Tris-HCl, 1 mM dithiothreitol, 125 μ M EDTA, 5% glycerol, pH 7.0), and centrifuged at 9,000 \times g for 10 min at 4°C. A portion of 50 μ g protein was incubated with 20 μ M Ac-DEVD-AFC (Calbiochem Inc.), a fluorescent substrate of caspase 3, in 500 μ l of the assay buffer (50 mM Tris-HCl, 1 mM EDTA, and 10 mM EGTA, pH 7.0) at 37°C for 30 min in the dark [7]. The fluorescence intensity of the reaction product was determined on a spectrofluorometer at the excitation wavelength of 380 nm and the emission wavelength of 510 nm.

2.7. Western blot analysis

Cells were incubated at 4°C for 15 min in a lysis buffer (4 mM EDTA, 2 mM EGTA, 1% Triton X-100, 50 mM HEPES, pH 7.8), and centrifuged at 1,700 \times g for 15 min at 4°C. The supernatant was collected and referred to as cell lysate. A portion of 50 μ g cell lysate was separated by SDS-PAGE and immunoblotted with indicated antibodies described above. After wash for 3 times with TBS containing 0.1% Tween 20, the membrane was incubated with the HRP-conjugated secondary antibodies (Sigma-Aldrich Chemical Co.) for 1 h at room temperature, and the signal was detected by using the ECL reagent (PerkinElmer Life Sciences, Waltham, MA).

2.8. Gene silencing by transient transfection of a siRNA expression plasmid

Human PKC δ and ERK1/2 were knocked-down in the cybrids by using the commercial siRNA expression plasmids pKD-PKC δ -v3 and pKD-MAPK1/Erk2-v5, respectively, and a negative control plasmid pKD-NegCon-v1 purchased from Millipore. The cybrids were plated at a density of 2.0×10^6 cells in 100-mm culture dishes. After culture for 24 h, transient transfection was performed with 20 μ g siRNA plasmid DNA and lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After transfection for 48 h, cells were subject to induction of apoptosis and biochemical assays.

2.9. Statistical analysis

Statistical analysis was performed by the Microsoft Excel 2003 statistical package. The results obtained from the cybrids are presented as mean \pm SD of the results from 3 independent experiments, and the data from the fibroblasts of different donors are presented as mean \pm SEM of the results from 3 independent experiments. The significant level of difference between the control and experimental groups was determined by Student's t test. A difference is considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Mitochondrial dysfunction and ROS overproduction in the $\Delta 4977$ cybrid

We chose the $\Delta 4977$ cybrid and the control harboring wild-type mtDNA (Fig. 1A) to examine the effects of a large-scale deletion of mtDNA on mitochondrial respiratory function and H_2O_2 production of the human cells. As shown in Fig. 1B, the oxygen consumption rate in the $\Delta 4977$ cybrid was decreased to 46% of that of the control. Moreover, the H_2O_2 production by the $\Delta 4977$ cybrid was about 1.7 fold of that of the control (Fig. 1C).

3.2. Activation of PKC δ and ERK1/2 by deleted mtDNA-elicited ROS accumulation

To understand the effect of H_2O_2 overproduction induced by 4977 bp-deleted mtDNA on signaling pathways, we measured the phosphorylation and protein levels of PKC δ and ERK1/2 under deleted mtDNA-elicited oxidative stress by Western blot. As shown in Fig. 2, the protein level of PKC δ was increased to about 1.8 fold in the $\Delta 4977$ cybrid, and the T507 phosphorylation of PKC δ (T505 in the mouse PKC δ) was increased to about 1.9 fold in the $\Delta 4977$ cybrid compared with those of the control. The phosphorylation level of ERK1/2 was increased to about 2.1 fold in the $\Delta 4977$ cybrid. However, there was no significant increase in ERK1/2 protein level in the $\Delta 4977$ cybrid compared with that of the control. Moreover, we observed that the phosphorylation levels of c-Raf, MEK1/2 and Elk-1 were also increased, although to different extents, in the $\Delta 4977$ cybrid (Fig. 2A).

3.3. Decrease of H_2O_2 and phosphorylation levels of PKC δ and ERK1/2 by NAC treatment

To confirm that deleted mtDNA-elicited oxidative stress could regulate the PKC δ and ERK1/2 signaling pathways, we treated the cybrids with the antioxidant NAC. The results showed that NAC effectively attenuated the H_2O_2 production of the $\Delta 4977$ cybrid in a dose-dependent manner (Fig. 3A). We also found that the levels of P-PKC δ , PKC δ and P-ERK1/2 were decreased in the $\Delta 4977$ cybrid that had been treated with 1 mM NAC (Fig. 3B).

3.4. Decrease in the susceptibility of the $\Delta 4977$ cybrid to UV-induced apoptosis by NAC

To examine whether deleted mtDNA-elicited oxidative stress is related to the susceptibility of the $\Delta 4977$ cybrid to UV-induced apoptosis, we measured the effect of NAC on caspase 3 activity in the cybrids upon UV irradiation. The results showed that 1 mM NAC could attenuate UV-induced caspase 3 activation in the $\Delta 4977$ cybrid (Fig. 3C).

3.5. Apoptotic susceptibility of the $\Delta 4977$ cybrid is due to ROS-elicited PKC δ activation

We then determined the role of PKC δ in the apoptotic susceptibility of the $\Delta 4977$ cybrid by a specific inhibitor, rottlerin, or by gene knockdown with siRNA of PKC δ . The results showed that 1 μ M rottlerin inhibited the PKC δ -elicited ERK1/2 phosphorylation in both cybrids, and was especially pronounced in the $\Delta 4977$ cybrid (Fig. 4A). Blocking the

activation of PKC δ by rottlerin led to the suppression of UV-induced caspase 3 activation in both cybrids, which was more effective in the Δ 4977 cybrid (Fig. 4C). Transient transfection with siRNA of PKC δ decreased the protein level of PKC δ , and the phosphorylation of ERK1/2 in the Δ 4977 cybrid to those of the control cybrid transfected with the NegCon vector (Fig. 4B). Moreover, suppression of PKC δ significantly attenuated the UV-induced caspase 3 activation in the Δ 4977 cybrid (Fig. 4D).

3.6. ERK1/2 phosphorylation is related to apoptotic susceptibility of the Δ 4977 cybrid

We also examined whether ERK1/2 is involved in the susceptibility of the Δ 4977 cybrid to UV-induced apoptosis by using a specific inhibitor, PD98059, or gene knockdown with siRNA of ERK1/2. Treatment of cybrids with 10 μ M PD98059 suppressed the phosphorylation of ERK1/2, but increased the protein levels of ERK1/2 in both cybrids (Fig. 5A). PD98059 treatment significantly decreased the UV-induced caspase 3 activation in the Δ 4977 cybrid to that of the control without treatment with PD98059 (Fig. 5C). In addition, transient transfection with siRNA of ERK1/2 decreased the ERK1/2 protein expression to about 50% of that transfected with the NegCon vector, and consequently reduced the phosphorylation levels of ERK1/2 in both cybrids (Fig. 5B). Furthermore, knockdown of ERK1/2 attenuated the UV-induced caspase 3 activation in the Δ 4977 cybrid (Fig. 5D).

3.7. *UV-induced apoptosis is mediated by caspase activation of PKC δ*

PKC δ is specifically digested by activated caspase 3 at the cleavage site in the hinge region to execute its apoptotic function [15]. We thus investigated whether the increased expression of PKC δ mediated by deleted mtDNA-elicited ROS can be used as an endogenous substrate of caspase 3 in the Δ 4977 cybrid to enhance the apoptosis induced by UV irradiation. Western blot analysis revealed that the amount of the ~17/20 kDa active caspase 3 was increased in both cybrids after UV irradiation, which was more pronounced in the Δ 4977 cybrid (Fig. 6, panel 4). As shown in Fig. 6, the protein level of the proteolytic cleavage form (~38/40 kDa) of PKC δ was notably accumulated in the Δ 4977 cybrid after UV irradiation. This was highly correlated with the activation of caspase 3.

3.8 *PKC δ and ERK1/2 activation in skin fibroblasts of CPEO patients*

To examine whether the PKC δ and ERK1/2 signaling pathways are activated in human cells harboring deleted mtDNA, we determined the phosphorylation and protein expression levels of PKC δ and ERK1/2 in skin fibroblasts from 3 CPEO patients harboring 4977 bp-deleted mtDNA and 3 age-matched normal subjects by Western blot, respectively. The average protein expression levels of P-PKC δ and P-ERK1/2 in skin fibroblasts of 3 CPEO patients were 2.0-fold and 3.8-fold higher than those of skin fibroblasts from 3

normal subjects, respectively. However, there were no differences in the average protein levels of PKC δ and ERK1/2 between the two groups of skin fibroblasts (Fig. 7).

4. Discussion

In this study, we demonstrated that H₂O₂ overproduction is associated with the susceptibility to UV-induced apoptosis of the Δ 4977 cybrid, which was effected by the activation of PKC δ and ERK1/2. Inhibition or knockdown of PKC δ and ERK1/2 could attenuate the UV-induced caspase 3 activation in the Δ 4977 cybrid. Moreover, we showed that 4977 bp deletion of mtDNA altered the PKC δ and ERK1/2 signaling pathways to enhance apoptotic responses as a result of oxidative stress elicited by mitochondrial dysfunction.

It has been well established that impairment of respiratory function and overproduction of ROS are the major causative factors of oxidative stress in human cells harboring a pathogenic mtDNA mutation [1]. The Δ 4977 cybrid showed a decrease of mitochondrial respiratory function and increase of H₂O₂ production (Figs. 1B and 1C), which are consistent with our previous findings [3]. As compared with the control, the mitochondrial respiratory function was reduced with the increase of 4977 bp-deleted mtDNA in the cybrids, and the content of ROS and the levels of oxidative damage (8-OHdG and malondialdehyde) were raised with the increase of the proportion of 4977 bp-deleted mtDNA in human cells [3,9]. These observations were confirmed by directly visualizing at single cell level through the application of fluorescent probes coupled with conventional and multiphoton imaging microscopy on the cybrids harboring 4977 bp-

deleted mtDNA [9]. The average production of ROS in fibroblasts from patients with CPEO syndrome was about 2-fold higher than that of control, and the mean 8-OHdG content in total DNA of muscle biopsies from three CPEO patients was about 9-fold higher than that of age-matched control [4]. Moreover, caspase 3 activation, TUNEL positive staining and overexpression of the pro-apoptotic protein Bax were found in the affected muscle fibers of CPEO, MERRF and MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) patients, respectively, who carried pathogenic mtDNA mutations [5,6]. Apoptosis was pronounced in the muscle from these patients and was correlated with abnormal proliferation of mitochondria (ragged-red fibers) [6]. In previous studies, we found distinct apoptotic features such as cytochrome *c* release, DNA fragmentation as well as caspase 3 activation in the cybrids harboring mutated mtDNA including point mutations and large-scale deletions upon treatment with H₂O₂, UV irradiation and staurosporine, respectively [7-9]. Moreover, point mutations of mtDNA associated with Leber's hereditary optic neuropathy (LHON) could also induce mitochondria-dependent apoptosis upon treatment with Fas, metabolic stress or oxidative stress [10,11,31]. These reports support the notion that pathogenic mtDNA mutations not only impair mitochondrial respiratory function and increase the production of ROS, but also augment the susceptibility to apoptosis of human cells. Furthermore, coenzyme Q₁₀ mitigated the UV-induced apoptosis in the cybrids harboring large-scale deletions of

mtDNA [12], and exogenous glutathione protected the *tert*-butyl hydroperoxide- or rotenone-treated LHON cybrids from cell death [31]. We observed that NAC significantly decreased the production of H₂O₂ in a dose-dependent manner (Fig. 3A), and effectively attenuated the UV-induced caspase 3 activation in the Δ 4977 cybrid (Fig. 3C). These findings suggest that deleted mtDNA-elicited ROS overproduction increases the sensitivity of the Δ 4977 cybrid to UV irradiation. It was found in our previous studies that high dose of UV irradiation could induce rapid and severe apoptosis in both control and Δ 4977 cybrids. However, we could not distinguish the extent of severity of apoptosis induced in these two cybrids by high dose of UV irradiation. This kind of acute cell death may be similar to the apoptosis triggered by angiotensin II-elicited generation of superoxide anions (about 7 fold in 3 h) and mtDNA damage, which could lead to apoptosis rapidly through the activation of mitochondrial permeability transition pores [32]. But, the clinical symptoms of mitochondrial disorders and neurodegenerative diseases usually progress with time and are related to long-term effects of low-level oxidative stress. We thus used the low-dose UV irradiation as an apoptotic trigger to examine the susceptibility to apoptosis of human cells harboring a pathogenic mutation of mtDNA, and focused on the long-term effects of 4977 bp-deleted mtDNA-elicited ROS overproduction on apoptosis.

On the other hand, we investigated the signaling mechanism involved in the apoptotic susceptibility of the Δ 4977 cybrid to UV irradiation. PKC δ plays a proapoptotic role in

mammalian cells exposed to various stimuli, and is considered an oxidative stress-responsive kinase [13,33]. In a previous study, we demonstrated that PKC δ was phosphorylated at threonine and tyrosine residues, and its protein level and kinase activity were increased by H₂O₂ treatment of human cells [23]. We showed in this study that the protein expression and phosphorylation levels of PKC δ were increased by overproduction of H₂O₂ in the Δ 4977 cybrid (Fig. 2). In addition, the decrease in H₂O₂ production by NAC treatment of the Δ 4977 cybrid (Fig. 3A) attenuated both oxidative stress-induced PKC δ activation (Fig. 3B) and deleted mtDNA-related caspase 3 activation upon UV irradiation (Fig. 3C). Furthermore, the role of PKC δ in the high susceptibility to UV-induced apoptosis was elucidated by using rottlerin and siRNA directed against PKC δ , respectively. We found that blocking of PKC δ signaling could mitigate the activation of caspase 3 in the Δ 4977 cybrid triggered by UV irradiation (Fig. 4). Moreover, we observed that the over-expressed PKC δ in the Δ 4977 cybrid could be used as a caspase 3 substrate to form a catalytic fragment to amplify the UV-induced apoptosis (Fig. 6). In addition to triggering the downstream signaling pathway via ERK1/2, the up-regulation and activation of PKC δ may serve as an amplifier for the mtDNA deletion-related susceptibility of UV-induced apoptosis of the Δ 4977 cybrid.

ERK1/2 is traditionally considered as a survival regulator to modulate proliferation and differentiation of mammalian cells, and can be activated by oxidative stress [24,25].

In recent years, a number of studies demonstrated that inhibition of ERK1/2 by PD98059 resulted in the resistance of mammalian cells to ROS-induced apoptosis [27,28,34], which revealed that ERK activation plays a role in the execution of apoptosis triggered by various stimuli [25]. In this study, we observed that ERK1/2 was significantly activated by H₂O₂ in the Δ 4977 cybrid (Fig. 2), and that NAC treatment decreased the phosphorylation of ERK1/2 (Fig. 3B). Moreover, UV irradiation-induced caspase 3 activation of the Δ 4977 cybrid was attenuated by PD98059 and siRNA of ERK1/2, respectively (Figs. 5C and 5D). These findings support the notion that the high sensitivity of the Δ 4977 cybrid to UV-induced apoptosis was conferred by mtDNA deletion-elicited ROS accumulation and ERK1/2 activation. The duration of ERK1/2 activation could turn on different sets of transcription factors or other downstream targets, which may in turn lead to diverse outcomes (e.g., proliferation, differentiation and apoptosis) of cells [26]. We conjecture that continual accumulation of ROS in the Δ 4977 cybrid may cause a chronic activation of ERK1/2, which render the mutant cells to be more sensitive to apoptotic stimuli.

Mitochondrial dysfunction, oxidative stress and apoptotic cell death have been implicated in mitochondrial diseases, neuromuscular disorders and neurodegenerative diseases [1,35]. Degeneration of muscle fibers and neuronal cell loss in affected tissues may be the result of enhanced apoptosis associated with mitochondrial dysfunction and the elevation of oxidative stress. We have demonstrated that the cybrids defective in the

mitochondrial respiratory function were more susceptible to apoptosis (Figs. 1B and 3C) [3,7], and that NAC (Fig. 3C) and coenzyme Q₁₀ [12] attenuated the UV-induced apoptosis in the cybrids harboring large-scale deletions of mtDNA. These observations indicate that the sensitivity of the Δ 4977 cybrid to UV irradiation was a result of deleted mtDNA-elicited ROS overproduction. However, it has remained unclear as to how the signaling pathways are involved in the susceptibility to apoptosis of human cells harboring mtDNA mutations and their role in the pathogenesis of degenerative diseases. Mitochondria-elicited oxidative stress and oxidative damage are accumulated in the affected brain regions during disease progression, which was suggested to play an important role in neuronal cell death [1,35]. Moreover, high levels of 4977 bp-deleted mtDNA have been found in the substantia nigra of elderly subjects [36] and in the affected brain regions of patients with frontotemporal dementia, Parkinson's disease (PD) [37] and dementia with Lewy bodies, respectively [38]. It has been shown that PKC δ and ERK1/2 are oxidative stress-sensitive kinases and key apoptotic mediators [33,39]. Several studies demonstrated aberrant activation of PKC δ and ERK1/2 in the cell culture and animal models of PD and amyotrophic lateral sclerosis and in brain tissues of patients with Alzheimer's disease (AD) or PD [39-41]. Blocking of PKC δ activation by kinase dominant negative mutant, cleavage-resistant mutant, siRNA or rottlerin effectively prevented dopaminergic cell death in both cell culture and animal models of PD [40]. In this study, we demonstrated that

PKC δ and ERK1/2 were activated by deleted mtDNA-elicited ROS (Figs. 2 and 3), which was involved in the susceptibility to UV-induced apoptosis of the cybrids harboring 4977 bp deletion of mtDNA (Figs. 4, 5 and 6). Moreover, we observed that the levels of activated PKC δ and ERK1/2 in skin fibroblasts of CPEO patients were higher than those of skin fibroblasts from normal subjects (Fig. 7). These results are consistent with the observations made in previous studies of PD and AD [39-41]. It will be of great interest to observe the activation status of PKC δ and ERK1/2 in the biopsies of affected tissues from patients with CPEO syndrome and other mitochondrial diseases. Since no drugs are currently available to prevent the progression of neurodegenerative disorders and mitochondrial diseases, our findings suggest that attenuation of oxidative stress by antioxidants may alleviate the symptoms and/or slow down the progression of these diseases. Moreover, both PKC δ and ERK1/2 may be considered as potential targets for the development of drugs for treatment or prevention of the progression of mitochondrial diseases and neurodegenerative diseases.

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List of abbreviations:

8-OHdG, 8-hydroxy 2'-deoxyguanosine; AD, Alzheimer's disease; CPEO, chronic progressive external ophthalmoplegia; H₂O₂, hydrogen peroxide; LHON, Leber's hereditary optic neuropathy; MAPK, mitogen-activated protein kinase; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibers; mtDNA, mitochondrial DNA; NAC, N-acetyl-L-cysteine; PD, Parkinson's disease; ROS, reactive oxygen species.

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Figure Legends

Fig. 1. Effects of 4977 bp deletion of mtDNA on the oxygen consumption rate and H₂O₂ production. (A) The proportion of 4977 bp-deleted mtDNA in the Δ 4977 cybrid was about 80% that was determined by Southern blot. About 5 μ g *Bam* HI-digested total DNA of each cybrid was separated by 0.8 % agarose gel, transferred to a piece of nylon membrane, and hybridized with digoxigenin-labeled mtDNA D-loop probe (D-loop region, np 16492 to np 466), and then quantified by a laser scanning densitometer. 16,569 bp DNA represents the wild-type mtDNA; and 11,592 bp DNA fragment represents the 4977 bp-deleted mtDNA. (B) The oxygen consumption rate of 1.0×10^6 cells under the basal condition was determined in 3 min by the 782 Oxygen Meter and SI 782 Oxygen System software. (C) H₂O₂ production from the cybrids under the basal condition was determined by using Amplex Red. After normalization with cell numbers, the fluorescence signal for the control was arbitrarily set at 100%. Each bar represents mean \pm SD of the results from 3 independent experiments. Asterisk: *, $p < 0.05$ vs. the control.

Fig. 2. Increase of the expression of ROS-elicited signaling molecules by 4977 bp-deleted mtDNA. (A) Western blot analysis of the expression levels and phosphorylation state of the members of the PKC δ -mediated c-Raf-MEK1/2-ERK1/2 signaling pathway in both

cybrids. GAPDH was a loading control. (B) Analysis of the protein levels of P-PKC δ (T507), PKC δ , P-ERK1/2 and ERK1/2 in both cybrids. The data represent mean \pm SD of the results from 3 independent experiments. Asterisk: *, $p < 0.05$ vs. the control.

Fig. 3. Protective effects of NAC on H₂O₂ production and UV-induced apoptosis in the Δ 4977 cybrid. (A) H₂O₂ production from the cybrids after treatment with different concentrations of NAC was determined by using Amplex Red. The fluorescence signal for the untreated control was arbitrarily set at 100%, as for Fig. 1C. (B) Total proteins were extracted from both cybrids following pre-treatment with or without 1 mM NAC, and were then subjected to Western blotting with indicated antibodies. GAPDH was a loading control. (C) The cybrids had been pre-treated with 1 mM NAC for 18 h before UV irradiation, and further incubated in fresh DMEM containing 1 mM NAC after exposure to UV irradiation. The caspase 3 activity was assayed by using DEVD-AFC as substrate. Each bar represents mean \pm SD of the results from 3 independent experiments. Asterisk: *, $p < 0.05$ vs. the untreated control; Dagger: †, $p < 0.05$ vs. without NAC treatment of the same cells.

Fig. 4. Depression of the sensitivity to UV-induced apoptosis of the Δ 4977 cybrid by PKC δ inhibition. Total cellular proteins were extracted from both cybrids in the presence

or absence of 1 μ M rottlerin (A) and transfected with NegCon vector or PKC δ siRNA (B), and were then subjected to Western blotting with indicated antibodies. GAPDH was a loading control. (C) The cybrids had been pre-treated with 1 μ M rottlerin for 18 h before UV irradiation, and were further incubated in fresh DMEM containing 1 μ M rottlerin after exposure to UV. The caspase 3 activity was determined by using DEVD-AFC as substrate. Asterisk: *, $p < 0.05$ vs. the control with DMSO treatment; Dagger: †, $p < 0.05$ vs. DMSO treatment of the same cells. (D) The cybrids were exposed to UV irradiation after being transfected with NegCon vector or PKC δ siRNA for 48 h. The caspase 3 activity was determined by using DEVD-AFC as substrate. Asterisk: *, $p < 0.05$ vs. the control with NegCon vector transfection; Dagger: †, $p < 0.05$ vs. NegCon vector transfection of the same cells. Each bar represents mean \pm SD of the results from 3 independent experiments.

Fig. 5. ERK1/2 phosphorylation induced by 4977 bp deletion of mtDNA was related to the sensitivity of the Δ 4977 cybrid to UV-induced apoptosis. (A) Total proteins were extracted from both cybrids in the presence or absence of 10 μ M PD98059, and then subjected to Western blotting with indicated antibodies. (B) Total proteins from both cybrids transfected with NegCon vector or ERK1/2 siRNA were subjected to Western blotting with indicated antibodies. GAPDH was a loading control. (C) The cybrids had been pre-treated with PD98059 for 18 h before UV irradiation, and were further incubated

in fresh DMEM containing 10 μ M PD98059 after exposure to UV. The caspase 3 activity was determined by using DEVD-AFC as substrate. Asterisk: *, $p < 0.05$ vs. the control with DMSO treatment; Dagger: †, $p < 0.05$ vs. DMSO treatment of the same cells. (D) The cybrids were exposed to UV irradiation after transfection with NegCon vector or ERK1/2 siRNA for 48 h. The caspase 3 activity was determined by using DEVD-AFC as substrate. Asterisk: *, $p < 0.05$ vs. the control with NegCon vector transfection; Dagger: †, $p < 0.05$ vs. NegCon vector transfection of the same cells. Each bar represents mean \pm SD of the results from 3 independent experiments.

Fig. 6. Amplification of UV-induced apoptosis through the caspase 3-mediated proteolytic activation of PKC δ . Total proteins were extracted from both cybrids after UV irradiation, and were then subjected to Western blotting with indicated antibodies. GAPDH was a loading control.

Fig. 7. Higher protein levels of P-PKC δ and P-ERK in skin fibroblasts of patients with CPEO syndrome. (A) Skin fibroblasts of 3 patients with CPEO syndrome and 3 age-matched normal subjects at passage 8 to 12 were grown to 80% confluence in 100-mm culture dishes in DMEM supplemented with 5% FBS. Total proteins were extracted and subjected to Western blotting with indicated antibodies. GAPDH was a loading control.

(B) Analysis of the protein levels of P-PKC δ , PKC δ , P-ERK1/2 and ERK by normalization with GAPDH in each group of the skin fibroblasts. The protein level of each signaling molecule in the skin fibroblasts was modified from the N1 donor, which was taken as 1. Each bar represents mean \pm SEM of the results from 3 independent experiments. Asterisk: *, $p < 0.05$ vs. normal subjects.

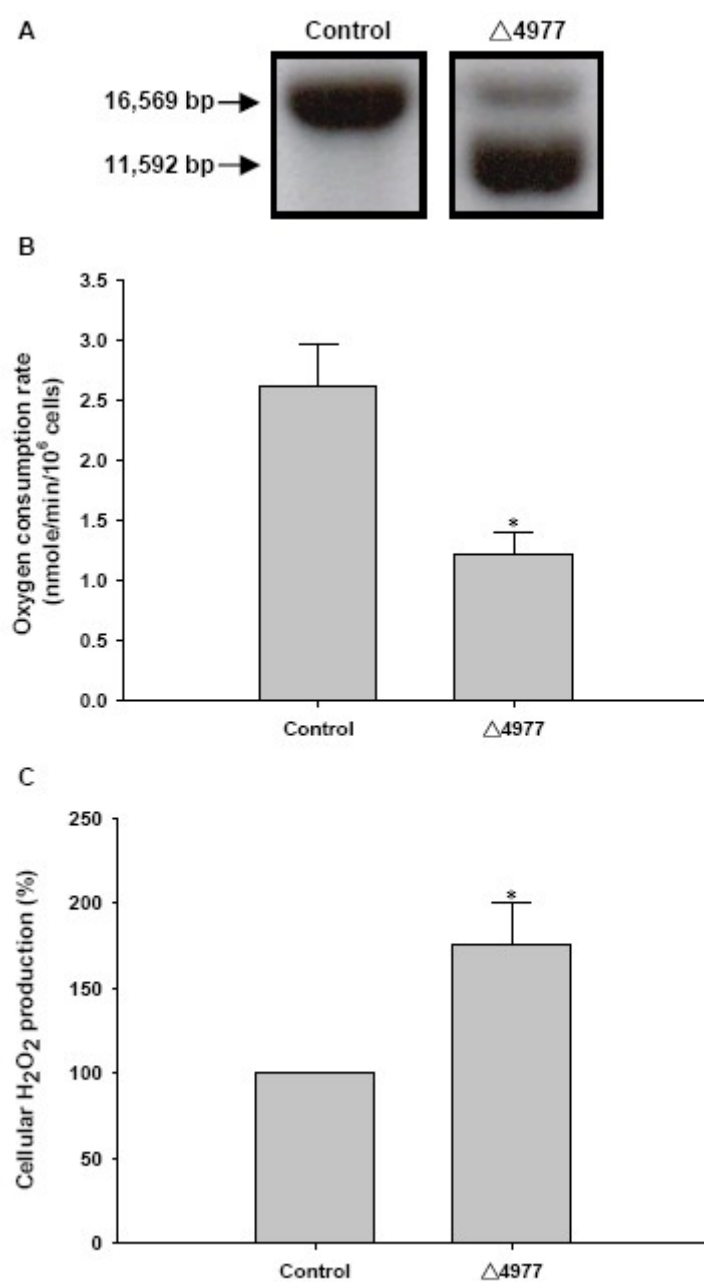


Fig. 1

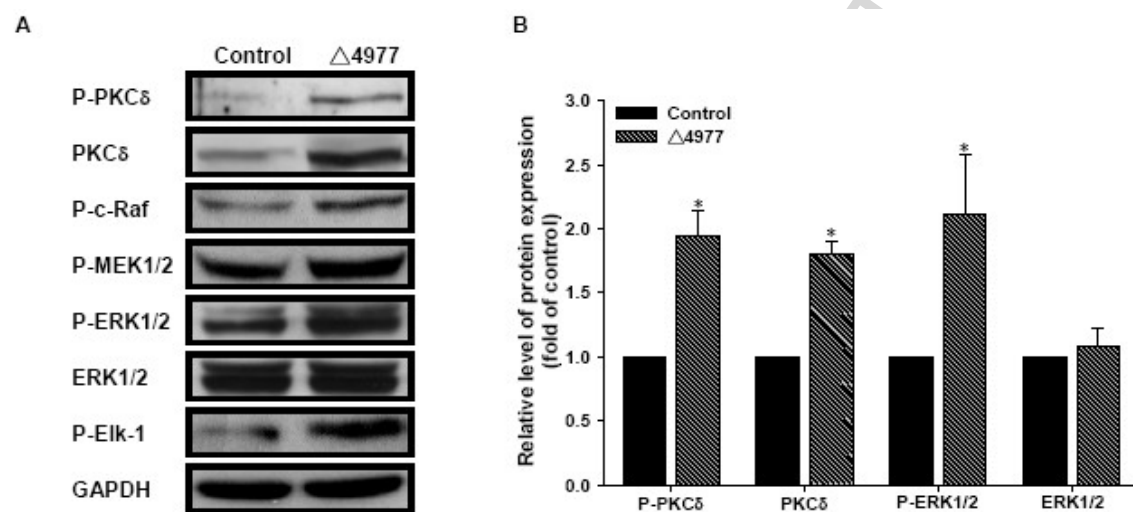


Fig. 2

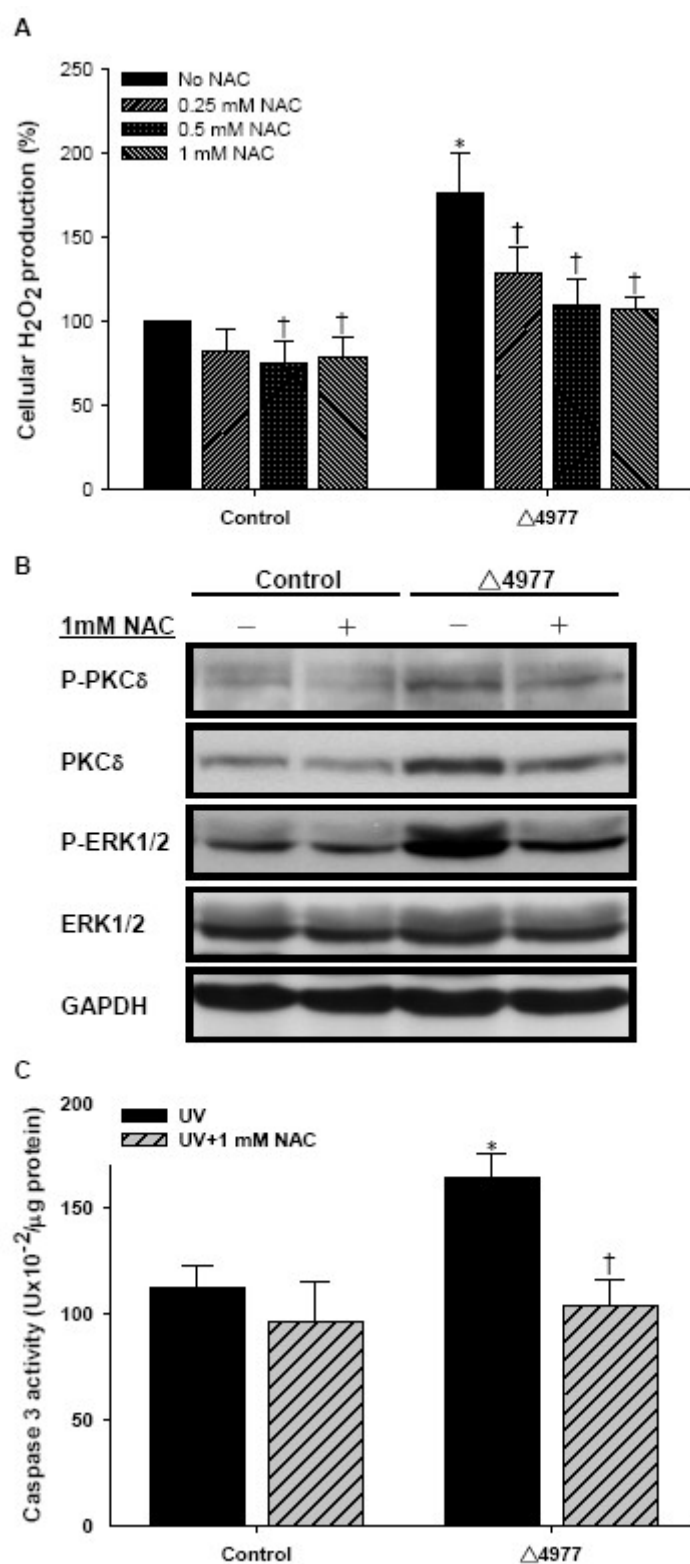


Fig. 3

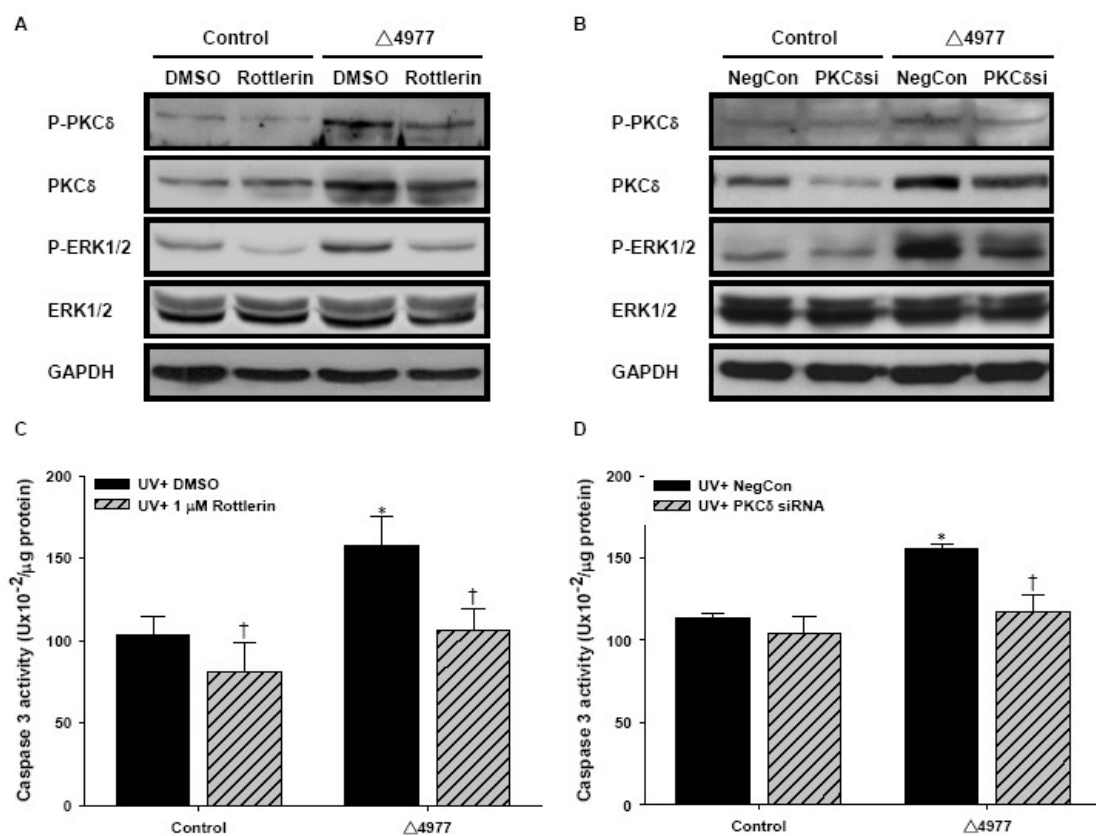


Fig. 4

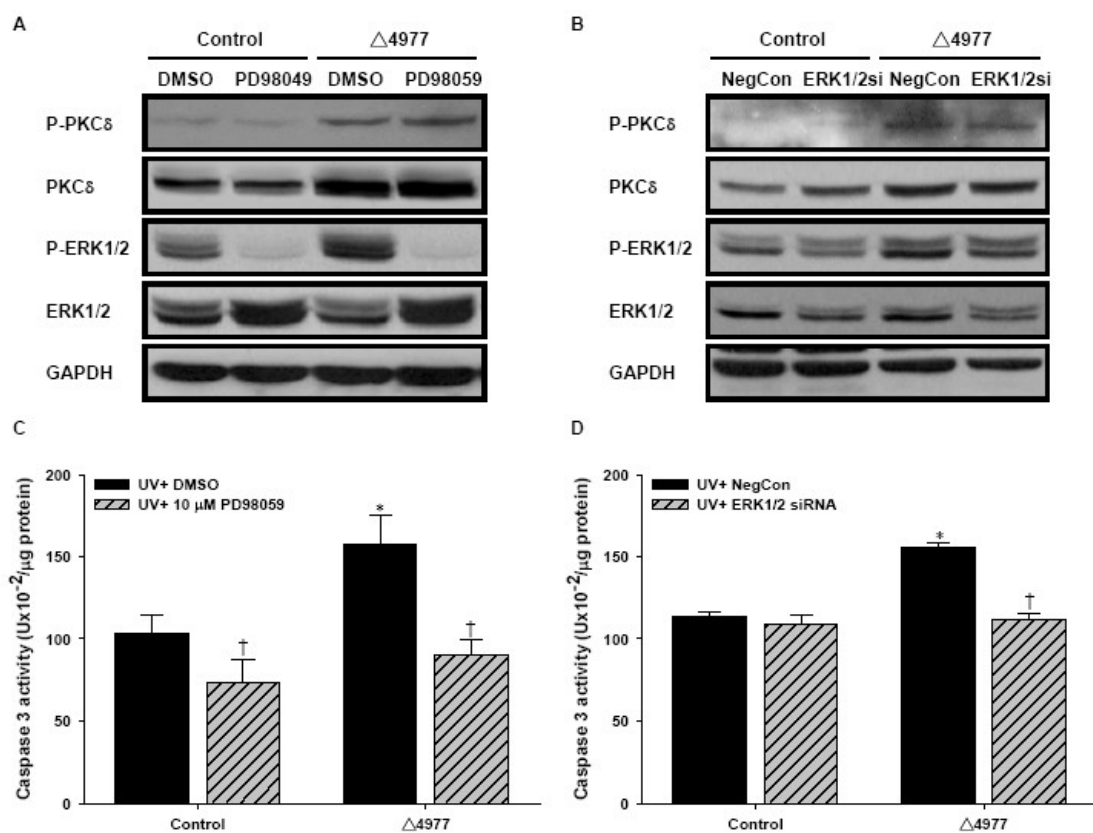


Fig. 5

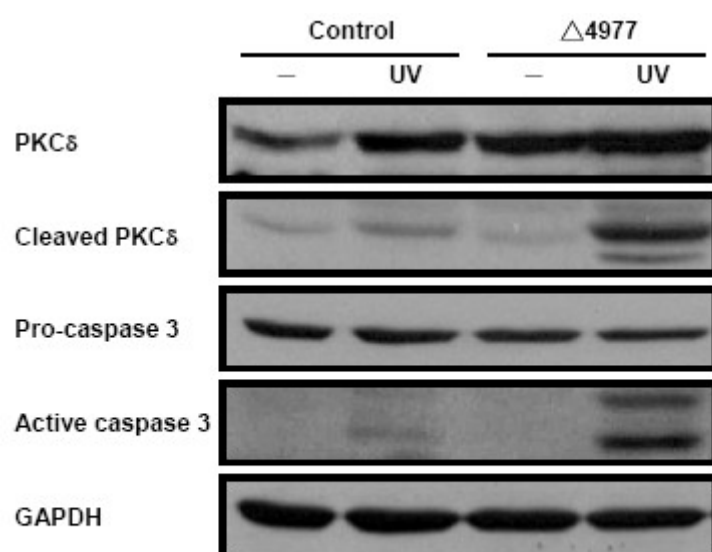


Fig. 6

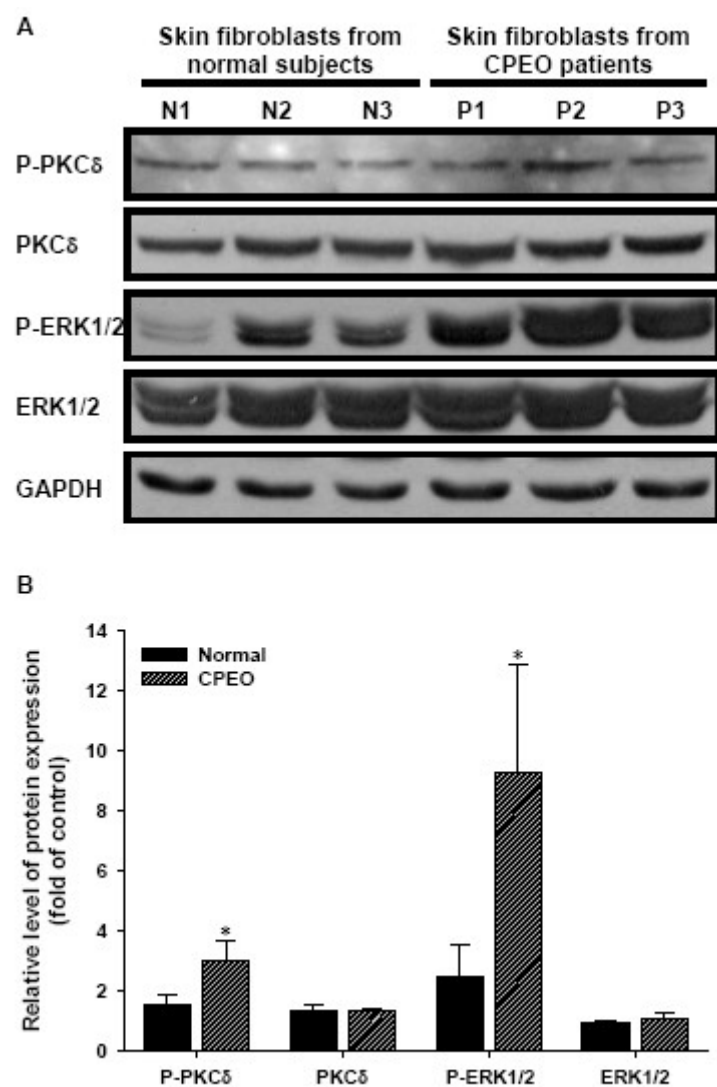


Fig. 7