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Paraquat induces apoptosis in human lymphocytes: Protective and rescue effects of glucose, cannabinoids and insulin-like growth factor-1

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Abstract

In order to establish causal or protective treatments for Parkinson's disease (PD), it is necessary to identify the cascade of deleterious events that lead to the dysfunction and death of dopaminergic neurons. Paraquat (PQ) is a pesticide used as xenobiotic compound to model PD. However, the mechanism(s) of PQ-induced cell death and the mechanism(s) of cytoprotection in a single cell model are still unknown. In this study, lymphocytes were treated with (0.1–1 mM) PQ. Apoptotic morphology was assessed with acridine orange/ethidium bromide staining. Further evaluation included (i) superoxide radicals, reflected by nitroblue tetrazolium reduction to formazan, (ii) the production of hydrogen peroxide, reflected by rhodamine-positive fluorescent cells, (iii) the generation of hydroxyl radicals, reflected by dimethylsulfoxide and melatonin $^{\cdot}OH$ scavengers, (iv) activation and/or translocation of NF-κB, p53 and c-Jun transcription factors showed by immunocytochemical staining, and by ammonium pyrrolidinedithiocarbamate, pifithrin-α and SP600125 inhibition and (V) caspase-3 activation, reflected by caspase Ac-DEVD-cho inhibition. To elucidate the mechanism of cytoprotection, lymphocytes were treated with PQ in the presence of cannabinoids, insulin-like growth factor-1 and glucose. We provide evidence that PQ induces apoptosis in lymphocytes in a concentration- and time-dependent fashion by an oxidative stress mechanism involving $O_2^{\cdot-}$, $H_2O_2/(^{\cdot}OH)$ generation, simultaneous activation of NF-κB/p53/c-Jun transcription factors, mitochondrial depolarization and caspase-3 activation leading to morphological apoptosis. Moreover, dying lymphocytes are protected and rescued from PQ noxious stimuli by direct antioxidant effect by cannabinoids, receptor mediated signaling by IGF-1, and/or energetic protection by glucose. It is concluded that PQ-induced apoptosis in lymphocytes by a mechanism involving reactive oxygen species generation, mitochondrial dysfunction, transcriptional factors and caspase-3 activation. However, this cell death routine can be reversed by the action of cannabinoids, IGF-1 and glucose. These data may provide innovating therapeutic strategies to intervene environmentally or genetically susceptible PD population to oxidative stress.

Keywords: *Apoptosis, c-Jun, oxidative stress, NF-κB, paraquat, p53*

Abbreviations: *AO, Acridine orange; Ac-DEVD-cho, Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Glu-Val-Asp-CHO; PDTC, ammonium pyrrolidinedithiocarbamate; JNK, c-Jun N-terminal kinase; DES, desipramine; DAB, diaminobenzidine; DHR, dihydrorhodamine; $D_iOc_6(3)$, 3,3′ dihexyloxacarbocyanine iodide; DMSO, dimethylsulfoxide; EB, ethidium bromide; H_2O_2, hydrogen peroxide; OH, hydroxyl radical; IGF-1, insulin-like growth factor-1; MEL, melatonin; $\Delta\Psi_m$, mitochondrial transmembrane potential; NAC,* N*-acetyl-cysteine; NBT, nitroblue tetrazolium; NF-κB, nuclear factor-kappa B; PQ, paraquat; PBL, peripheral blood lymphocytes; PFT, pifithrin-α; SP600125, 1,9-pyrazoloanthrone; ROS, reactive oxygen species; $O_2^{\cdot-}$, superoxide anion radical*

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta region of the midbrain. Although the cause of all cases of PD remains unknown, epidemiological evidence has suggested that genetic susceptibility (Farrer 2006; Tan and Skipper 2007) as well as

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environmental factors (Di Monte et al. 2002), specially pesticides (Dick 2006) may be responsible for the associated neurodegeneration in PD. Indeed, paraquat dichloride (PQ, methyl viologen dichloride), or 1,1′-dimethyl-4,4′-bipyridinium dichloride, a widely used non-selective herbicide, has been suggested as a prime risk factor for the disease (Dinis-Oliveira et al. 2006a,b) primarily due to PQ exposure (Morano et al. 1994; Liou et al. 1997) and due to its similarity to the chemical structure of the active metabolite of the parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridium ion (MPP^+). PQ has been shown to be highly toxic to both neuronal and non-neuronal cells mainly because its reduction–oxidation cycling reaction capability between its ionic and radical forms appears to induce conversion of molecular oxygen to the superoxide anion and to other reactive oxygen species (ROS). Biochemically, PQ (dication, PQ^{2+}) is reduced by cellular reductases such as NAD(P)H cytochrome P-450 reductase (EC 1.6.2.4) and NADH: ubiquinone oxidoreductase (1.6.5.3; also known as mitochondrial complex 1 or NADH dehydrogenase), to form a PQ monocation free radical ($PQ^{\cdot+}$). This last radical is then rapidly reoxidized in the presence of oxygen generating the superoxide anion radical ($O_2^{\cdot-}$). This then sets off the well-known enzymatic or non-enzymatic dismutation of $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2) which in the presence of ferrous iron (Fe^{2+}) is capable of forming the highly reactive and harmful hydroxyl radical ($^{\cdot}OH$). Thus, oxidation of PQ leads to an overproduction of ROS, oxidative stress (defined as an intracellular overload production of ROS which are not balanced by an adequate antioxidant system), and mitochondrial impairment and cell death. Recently, it has been demonstrated that PQ induces dopaminergic nigral apoptosis—a type of programmed cell death—in both mesencephalic dopaminergic neuronal N27 and primary rat hippocampal E18 cell lines, and mouse by sustained activation of the c-Jun N-terminal kinase (JNK) and c-Jun (Peng et al. 2004; Niso-Santano et al. 2006; Klintworth et al. 2007). Yet, the complete mechanism(s) of cell death signalization induced by PQ in a single cell model is still unknown.

In order to establish causal or protective treatments for PD, it is necessary to identify the cascade of deleterious events that lead to the dysfunction and death of dopaminergic neurons. PQ has been extensively used as xenobiotic compound to model PD (Bove et al. 2005). As PQ is chemically a redox cycling molecule and H_2O_2-generator, it is conceivable that PQ might induce a molecular cell death mechanism analogous to other relatively well known redox cycling neurotoxins such as 6-hydroxydopamine, and 5,6-dihydroxytryptamine and toxic peptides such as $A\beta_{(25-35)}$ shown to induce apoptosis in lymphocytes through H_2O_2-induced impairment of mitochondrial transmembrane potential ($\Delta\Psi_m$), activation pro-apoptotic transcription factors such as NF-κB, p53, c-Jun; and protease caspase-3 activation (Jimenez-Del-Rio and Velez-Pardo 2002; Jimenez-Del-Rio et al. 2004). To test this hypothesis, we sought (i) to investigate whether PQ induces apoptosis in lymphocyte cells, which are used as non-neuronal system to model mechanistic pathways in oxidative stress-induced cell demise associated to neurodegeneration (Jimenez-Del-Rio and Velez-Pardo 2004), (ii) to determine whether PQ induces oxidative stress through H_2O_2, and activation of pro-apoptotic transcription factors and (iii) to examine whether PQ activates the pivotal executor of apoptosis, caspase-3.

The insulin-like growth factor-1 (IGF-1), which is a polypeptide with high sequence similarity to insulin, and the cannabinoids, which are a group of terpenophenolic compounds present in Cannabis (*Cannabis sativa* L.), have been postulated to be neuroprotective agents because of their observed capacity to prolong the survival or preserve the function of neurons (Kurmasheva and Houghton 2006; Lastres-Becker and Fernandez-Ruiz 2006; Davila et al. 2007). Recently, we have demonstrated that IGF-1 and the synthetic cannabinoids JWH-015 and CP55,940 were able to protect and rescue lymphocytes against H_2O_2-induced apoptosis by receptor-independent (i.e. cannabinoids) and receptor-dependent (i.e. both IGF-1 and cannabinoids) mechanisms (Jimenez-Del-Rio and Velez-Pardo 2006; Velez-Pardo and Jimenez-Del-Rio 2006). Therefore, we further examine (iv) whether pre-treatment of cells with natural [e.g. IGF-1, melatonin (MEL)] or synthetic cannabinoid agonist JWH-015 and CP55,940 compounds may protect and rescue peripheral blood lymphocytes (PBL) against PQ-toxicity. Understanding the mechanism of PQ-induced apoptosis may provide insights into more effective therapeutic approaches for PD.

Material and methods

Materials

If not otherwise specified, reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and were of analytical grade or of better grade. 3,3′-dihexyloxacarbocyanine iodide ($D_iOc_{(6)}3$, Cat. #D-273) and dihydrorhodamine (DHR, cat. #D-633) were obtained from Invitrogen Molecular Probes (Eugene, OR, USA). Caspase-3 inhibitor (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Glu-Val-Asp-CHO; Ac-DEVD-cho, Cat # 260-046-M001) was from Alexis Biochemical

Corporation (San Diego, CA, USA). Recombinant human IGF-1 was acquired from ProSpec-Techno-Gene (Weizmann Science Park, Rehovot, Israel). Ammonium pyrrolidinedithiocarbamate (PDTC, cat. # 548000) and 1,9-pyrazoloanthrone (SP600125, cat # 420119) were acquired from Calbiochem (San Diego, CA, USA).

Isolation of lymphocytes

PBL from venous blood of healthy adult male (range age 30–40 years old) were obtained by gradient centrifugation (lymphocyte separation medium, density: 1.007 G/M; Bio-Whittaker, Walkersville, MD, USA). Isolated PBL were washed three times with phosphate-buffered saline (PBS) (10 mM sodium phosphate, 160 mM NaCl, pH 7.4) and finally suspended in RPMI 1640 (GIBCO laboratories, NY, USA) plus 10% foetal calf serum (FCS, GIBCO laboratories). The PBL in suspension were cultured in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The PBL were plated in 24-wells (1×10^6 cells/ml/well).

Experiments with PBL

Assessment of cell death by fluorescent microscopy analysis using AO/EB double staining. Cell suspension (1 ml, final volume) was exposed to increasing concentration of PQ (0.1; 0.25; 0.5; 1, 3, 5, 10 mM PQ) freshly prepared in RPMI-1640 medium in absence or presence of different products of interest for 24 h at 37°C. Then, PBL were used for fluorescent microscopy analysis. After treatment, cells were mixed with 1 μl acridine orange (100 μg/ml AO final concentration) and ethidium bromide (100 μg/ml EB final concentration) and 5 μl was placed onto a slide and examined under fluorescence microscope (Zeiss Axiostart 50). Based on the differential uptake of the fluorescent DNA binding dyes AO/EB, normal PBL cells (bright green chromatin) can be discriminated from early apoptotic cells (bright green highly condensed or fragmented chromatin), late apoptotic cells (bright orange highly condensed or fragmented chromatin) and necrotic cells (bright orange/red chromatin). Quantification of apoptotic morphology was done by counting a minimum of 300 total cells as follows: % apoptotic cells = 100 × (total number of early and late apoptotic cells/total number of cells). Necrotic cells were not detected under the present experimental conditions. Apoptotic cells were microphotographed using a Zeiss (Axiostart 50) microscope equipped with a Zeiss MC80DX camera. Assessment of apoptotic indexes was repeated three times in independent experiments.

Determination of intracellular ROS

Assessment of superoxide anion radical generation. Because high concentrations PQ-induced massive clustering of cells, lymphocytes (1 ml, final volume) were exposed to (1 mM) PQ at 0, 1, 3, 6, 12 and 24 h. Then, cells were incubated with nitroblue tetrazolium (NBT, 1 mM final concentration) for 1 h. NBT is an electrophilic dicationic compound, which can easily accept electrons from electron donors (e.g. $O_2^{\cdot -}$). In the oxidized form, NBT chloride is a yellow compound soluble in aqueous medium. Its reduction to formazan is accompanied by disappearance of the positive charges, resulting in a substantial decrease in solubility, and the appearance of intense blue–purple color precipitate (Auclair and Voisin 1985). After incubation time, cells were examined for formazan formation. Quantification of formazan was done by counting a minimum of 250 total cells as follows: % formazan positive cells = 100 × (total number of blue color cells/total number of cells). Assessment was repeated three times in independent experiments. Positive formazan (blue–purple precipitate) stained cytoplasm was microphotographed using a Zeiss (Axiostart 50) microscope equipped with a Canon PowerShot G5 digital camera.

Assessment of hydrogen peroxide. Detection of H_2O_2 in cellular systems can be obtained by the use of the sensitive uncharged and nonfluorescent dihydrorhoramine 123 (DHR) as described elsewhere (Rothe and Valet 1994). Briefly, PBL (1×10^6 cells/ml) were incubated with 1 mM PQ and other products of interest. To evaluate H_2O_2 generation, aliquots of 100 μl untreated and treated PBL were incubated in the presence of 1 μM DHR (20 mM stock solution in DMSO) for 45 min at 37°C. DHR is oxidized to the cationic green fluorescent dye rhodamine-123 which accumulated intracellularly owing to the electrically negative cytoplasmic and mitochondrial membrane potential. The quantification of fluorescent cells under a fluorescence microscope (Zeiss Axiostart 50) was performed by counting a minimum of 300 total cells as follows: % fluorescent H_2O_2 cells (observed as completely green bright fluorescent cells) = 100 × (number of green fluorescent cells + scarce cytoplasmic fluorescent mitochondrial dots)/total number of cells (green fluorescent cells + non-fluorescent cells) compared to untreated control. Experiments were performed in three separate and independent settings. Parallel to $O_2^{\cdot -}$ and H_2O_2 evaluations, the percentage apoptotic cell were examined according to AO/EB staining assay.

Assessment of mitochondrial transmembrane potential ($\Delta\Psi_m$). PBL were treated as described above. PBL were incubated for 15 min at 37°C with the cationic

lipophilic $D_iOc_6(3)$ (1 μM, final concentration) to evaluate $\Delta\Psi_m$. The quantification of fluorescent cells (reflecting high membrane potentials) under the fluorescence microscope was performed by counting a minimum of 300 total cells as follows: % $D_iOc_6(3)$ fluorescent cells = 100 × (number of fluorescent cells)/total number of cells (non-fluorescent cells + fluorescent cells, reflecting high normal membrane potentials) compared to untreated control. Experiments were performed in three separate and independent settings.

Immunocytochemistry detection of NF-κB, p53 and c-Jun transcription factor proteins

Immunocytochemistry was performed according to suppliers' protocol (Santa Cruz Biotechnology, goat ABC staining System cat # sc-2023) using the primary goat poly-clonal antibodies NF-κB p65 (C-20)-G (Santa Cruz Biotechnology cat #sc-372-G), p53 (FL-393) (Santa Cruz Biotechnology cat #sc-6243-G) and p-(Ser 73)-c-Jun (Santa Cruz Biotechnology cat #sc-7981). After treatments, cells were plated on poly-L-lysine coated cover slip and fixed in 4% methanol in 0.1 M phosphate buffer, pH 7.4 for 25 min and then washed with PBS. Slides were exposed to 1% hydrogen peroxide in PBS for 10 min. After several washes, cells were permeabilized with Triton X-100 solution in PBS for 5 min. Cells were incubated with primary antibodies (10 μg/ml) for 2 h at room-temperature (RT) and subsequently incubated with biotinylated antibody at RT for 1 h. Finally, the specimens were stained with the ABC enzyme kit. After staining, they were cover-slipped with cover glasses. Positive diaminobenzidine (DAB) stained nuclei (dark-brown colour) were microphotographed using a Zeiss (Axiostart 50) microscope equipped with a Canon PowerShot G5 digital camera.

Natural or artificial antioxidant compounds, IGF-1 and glucose rescue experiments against PQ

PBL were pre-incubated with 1 mM PQ for 6 h at 37°C. Afterwards, cells were exposed to compounds listed in Table IV for 24 h. After this time, treated PBL were evaluated for apoptotic morphology as described above.

Statistical analysis

Data are means ± SD of three independent experiments. A difference between two groups was statistically analyzed by the Student's *t*-test. A *p*-value of <0.05 vs. control was considered significant.

Results

PQ induces apoptosis in lymphocytes through anion superoxide radical ($O_2^{\cdot-}$)/H_2O_2 generation, and mitochondrial damage

To examine whether PQ induces apoptosis in lymphocytes, PBL were incubated with increasing concentrations of PQ ranging from 0.1 to 10 mM at 37°C for 24 h. As shown in Figure 1(A), PQ induces apoptosis in a concentration-dependent fashion up to 1 mM (33% of apoptosis). In fact, treated cells exhibited many characteristics of apoptosis including cell shrinkage and rounding, chromatin condensation and/or nuclei fragmentation into ring-, and half-moon-like forms (Figure 1(B)) as evaluated by the conventional AO/EB staining technique (Leite et al. 1999). However, higher PQ concentrations (e.g. 3 up to 10 mM) induced massive clustering of cells avoiding a clear-cut evaluation of apoptotic morphology (data not shown). Therefore, 1 mM PQ was selected for further experiments.

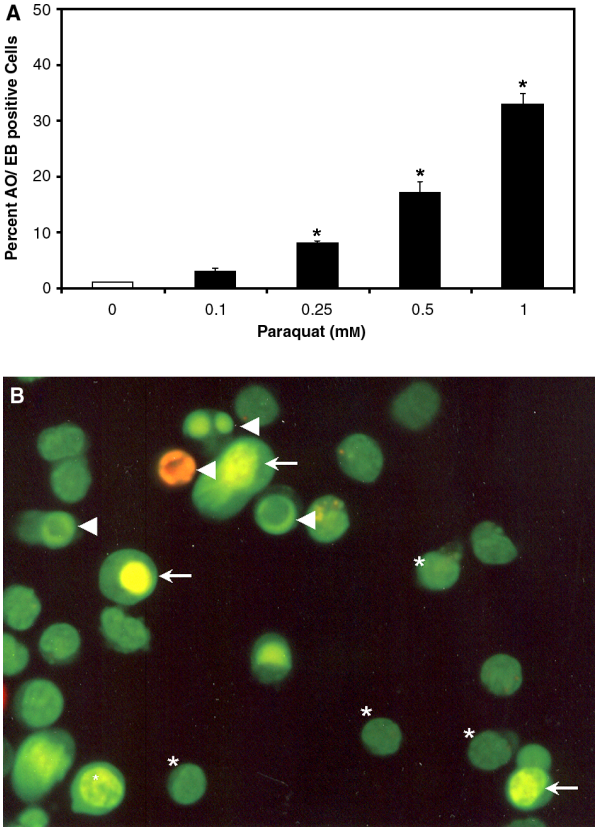

Figure 1. The herbicide PQ induces apoptosis in lymphocytes. (A) Lymphocytes were incubated with increasing concentrations of PQ for 24 h. The evaluation of apoptosis was performed as described in *Materials and methods*. The amount of apoptosis is expressed as mean of percentage + SD from three independent experiments. Photomicrograph (B) illustrates the typical nuclear apoptotic morphology such as highly condensed chromatin (*arrows*) and nuclear fragmentation (*arrowheads*) from lymphocytes treated with 1 mM PQ for 24 h. Asterisk represent normal cells. Picture represents one out of three independent observations. Magnification 400 × .

Time-course of PQ-induced toxicity. To investigate whether PQ generates ROS, and whether it induces impairment of mitochondria potential, lymphocytes were exposed to 1 mM PQ at 1, 3, 6, 12 and 24 h or left untreated for 24 h. Then, $O_2^{\cdot-}$ radicals/H_2O_2 generation, and mitochondria depolarization were assessed by reduction of NBT into formazan assay, oxidation of DHR into fluorescent rhodamine, and $D_iOC_6(3)$ assay. At the same time, the apoptotic morphology was evaluated by AO/EB staining. As illustrated in Figure 2(A), PQ (1 mM) induces the formation of cytoplasmic blue/purple colour precipitate (formazan) surrounding the nucleus (*arrowheads*), indicative of $O_2^{\cdot-}$ radical generation. Noticeably, 60–68% positive formazan cells were detected over a period of 1 h up to 6 h of PQ incubation. The percentage of positive formazan cells was moderately reduced from 23 to 16% at 12 and 24 h of PQ exposure (Figure 2(B)). H_2O_2 generation was detected as early as 1 h of PQ incubation and its percentage of production was gradually decreasing over time after a peak of generation at 3 h (Figure 2(B)). On the other hand, PQ induces a slight impairment of mitochondria transmembrane potential at 6 h, a moderate $\Delta\Psi_m$ depolarization effect is only observed after 12 h of poison incubation ($\Delta\Psi_m = 30\%$ $D_iOC_6(3)$ negative cells or depolarized cells), but this impairment is more severe at 24 h ($\Delta\Psi_m = 49\%$ $D_iOC_6(3)$ negative cells). Parallel examination of apoptotic morphology showed that AO/EB percentages increased progressively from 6 to 24 h PQ exposure (13, 22 and 33% AO/EB nuclei staining, Figure 2(B)).

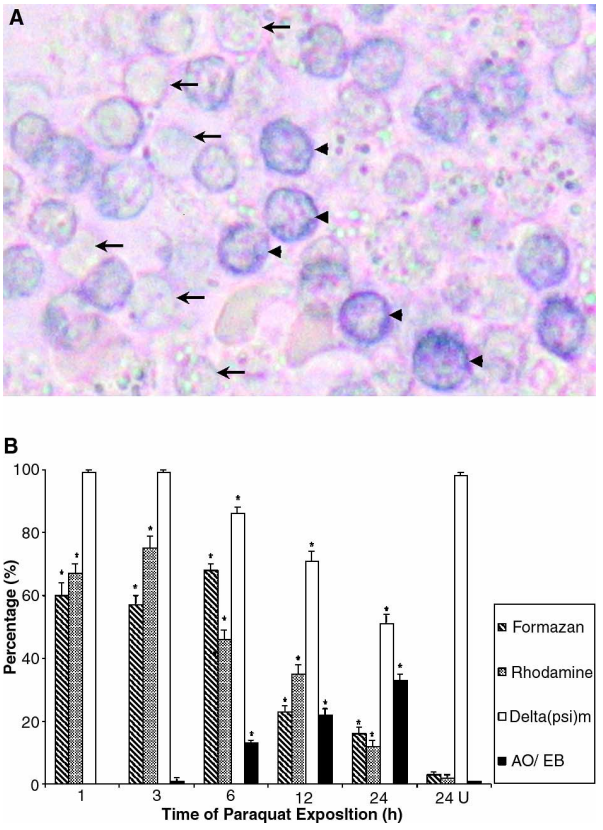

Figure 2. PQ produces ROS, mitochondrial damage and apoptosis in lymphocytes. Lymphocytes were pre-incubated with 1 mM PQ for 6 h and were evaluated for superoxide anion radicals and H_2O_2 production, and mitochondrial depolarization as described in *Materials and methods*. Photomicrograph (A) illustrates the typical NBT stained blue–purple precipitate cells (i.e. formazan, *arrowheads*) as positive $O_2^{\cdot-}$ generation, and NBT negative stained cells (i.e. translucent cells, *arrows*). (B) Lymphocytes were incubated with 1 mM PQ at different interval of time (h). The amount of $O_2^{\cdot-}/H_2O_2$ generation, $\Delta\Psi_m$ and apoptosis is expressed as mean of percentage + SD from three independent experiments. $\star p < 0.05$ vs. treated cells with PQ for 1 h as determined by the Student's *t*-test. Magnification (A) 600 ×.

PQ induces apoptosis in lymphocytes associated with NF-κB, p53 and c-Jun transcription factors and caspases-3 activation

Given that the transcription factors NF-κB (Radhakrishnan and Kamalakaran 2006), p53 (Chipuk and Green 2006) and c-Jun (Shen and Liu 2006) and the protease caspase-3 (Fan et al. 2005) have been involved in apoptosis signalling, we asked whether PQ induces the activation of these molecules in lymphocytes. Thus, cells were pre-incubated one hour with PDTC (10 nM, an inhibitor of NF-κB), PFT (50 nM, a specific inhibitor of p53), SP600125 (1 μM, a specific inhibitor of JNK), Ac-DEVD-cho (10 μM, a specific inhibitor of caspase-3) prior to incubation with 1 mM PQ for 24 h. As shown in Table I, all the specific pharmacological inhibitors reduced almost completely the PQ-induced apoptotic effect to control

Table I. NF-κB, c-Jun and p53 are signaling molecules involved in PQ-induced apoptosis.

Treatment/assay	AO/EB (%)	$D_iOc_6(3)$ (%)
Untreated	$<1 \pm 0$	$>99 \pm 0$
PQ (1 mM)	$33 \pm 2\star$	$60 \pm 2\star$
PDTC (10 nM)	$<1 \pm 0$	$>99 \pm 0$
PDTC (10 nM) + PQ (1 mM)	$<1 \pm 0$	$>99 \pm 0$
PFT (50 nM)	$<1 \pm 0$	$>99 \pm 0$
PFT (50 nM) + PQ (1 mM)	$<1 \pm 0$	$>99 \pm 0$
SP600125 (1 μM)	3 ± 1	98 ± 1
SP600125 (1 μM) + PQ (1 mM)	4 ± 1	97 ± 1
DEVD (10 μM)	$<1 \pm 0$	$>99 \pm 0$
DEVD (10μM) + PQ (1 mM)	$<1 \pm 0$	$>99 \pm 0$

Cells were left untreated or treated with specific NF-κB-, p53 and caspase-3 inhibitors, ammonium pyrrolidinedithiocarbamate (PDTC, 10 nM), pifithrin-α (PFT, 50 nM), SP600125 (1 μM) and Ac-DEVD-cho (DEVD, 10 μM) alone or in presence of PQ (1 mM) for 24 h. After this time, nuclear morphologic changes indicative of apoptosis as a result of NF-κB-, p53 caspase-3 activation were evaluated using AO/EB staining as described in *Materials and methods*. Apoptotic values are expressed as mean of percentage (%) + SD from two independent experiments. $\star p < 0.05$ vs. respective control as determined by the Student's *t*-test.

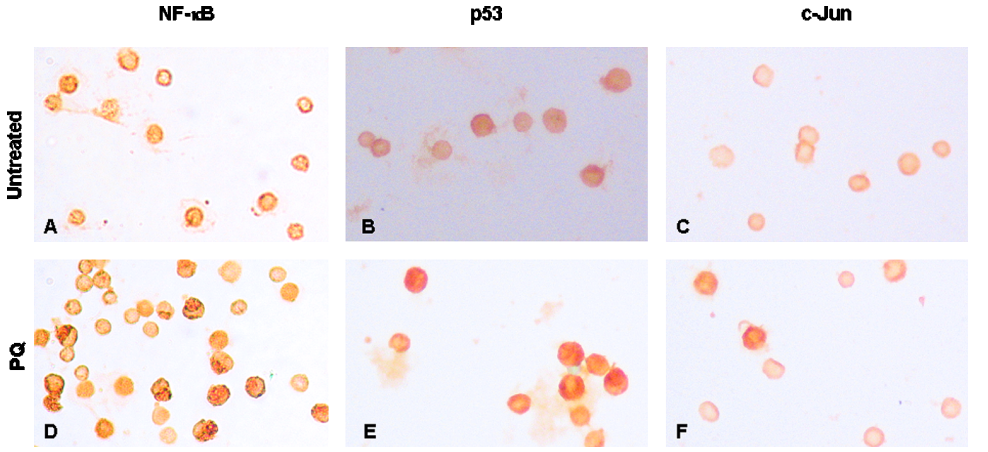

Figure 3. PQ induces simultaneous activation of the transcription factors in lymphocytes. PBL cells were left untreated (A–C) or exposed to 1 mM PQ (D–F) for 24 h. After this time of incubation, cells were stained with anti-NF-κB-p65 (A and D), anti-p53 (B and E) and anti-c-Jun (C and F) antibodies according to procedure described in *Materials and methods*. Notice that NF-κB, p53 and c-Jun positive-nuclei (dark brown colour) reflect their nuclear translocation/activation and appear to correlate with the apoptotic nuclear morphology, i.e. condensed/fragmented nuclei when compared with untreated cells (A–C). Magnification 400 × (A–F).

values (<1% AO/EB staining). To confirm the participation of NF-κB, p53 and c-Jun in PQ-induced apoptosis, we performed immunocytochemical assessment. As illustrated in Figure 3(D)–(F), cells incubated with 1 mM PQ clearly showed DAB-positive nuclei staining of the active form of NF-κB (15%), p53 (26%) and c-Jun (6%) as compared to untreated cells (0%), where inactive transcription factors reside in the cytoplasm (Figure 3(A)–(C)).

Lymphocytes are protected against PQ-induced cell death by glucose, IGF-1 and antioxidants

The above observations prompted us to test whether molecules involved in cellular metabolism, and/or antioxidant activity could protect lymphocytes against PQ toxicity. Then, cells were pre-treated with glucose (54 mM), *N*-acetyl-cysteine (NAC, 1 mM), vitamin C, E (0.5 mM), CP55,940 (100 nM), JWH-015 (100 nM), IGF-1 (100 nM) and desipramine (DES, 100 nM) for 1 h at 37°C. After this time of incubation, treated cells were co-incubated with 1 mM PQ for 1 and 24 h, and then evaluated for apoptotic morphology by AO/EB staining technique, mitochondrial damage by $D_iOc_6(3)$ assay, and $O_2^{\cdot-}$ radical generation by NBT assay. Table II shows that all compounds proved to be highly effective in protecting lymphocytes from PQ-induced apoptosis and mitochondrial damage at any interval of time. Interestingly, while the antioxidant compounds NAC, CP55,940, JWH-015, vitamin C, E and the monoamine transport inhibitor DES markedly blocked formazan formation to control values (<1% formazan positive cells), the formazan precipitate was evidently observed under glucose and IGF-1 exposure either at 1 or at 24 h.

Hydroxyl radicals are involved in PQ toxic effect on lymphocytes

It is accepted that aerobic incubation of PQ resulted in the production of $^{\cdot}OH$ (Zang et al. 1995). Therefore, we evaluated whether reactive $^{\cdot}OH$ could be implicated in the PQ-evoked cytotoxicity in lymphocytes. As shown in Table III, incubation of PBL cells with (1 mM) PQ in the presence of the $^{\cdot}OH$ scavengers dimethylsulfoxide (DMSO) and MEL moderately or completely blocked the apoptotic process and mitochondrial depolarization, respectively, when compared to PQ treatment alone. Moreover, DMSO and MEL moderately reduced the percentage of apoptosis and mitochondrial damage induced by the combination of H_2O_2 and Fe^{2+} (i.e. Fenton reaction) treatment, used as positive control. Noticeably, Fe^{2+} neither promotes apoptosis when co-incubated with PQ nor affects the protective effect of DMSO and MEL.

Natural or synthetic molecules are able to rescue lymphocytes from PQ induced cell death

As shown above, a significant percentage of apoptosis in lymphocytes is recognizable at 6 h of incubation when compared to 1 or 3 h of PQ exposure (Figure 2(B)). Therefore, we set up this interval of time to test whether the antioxidants (Vit C, Vit E, CP55,940, JWH-015 and MEL), glucose, IGF-1, and the signaling inhibitors PFT, Ac-DEVD-cho, PDTC were able to rescue lymphocytes against PQ toxicity. Interestingly, all molecules were either moderately (e.g. glucose, vitamin C) or completely effective in rescuing cells from PQ toxicity (Table IV).

Discussion

Oxidative stress has long been suggested to play a key role in the neurodegenerative process underlying PD

Table II. Metabolic and antioxidant molecules protect lymphocytes against PQ toxicity.

Incubation time	1 h			24 h		
Treatment/assay	AO/EB (%)	$D_iOc_6(3)$ (%)	Formazan (%)	AO/EB (%)	$D_iOc_6(3)$ (%)	Formazan (%)
Untreated	0	$>99 \pm 0$	0	$<1 \pm 0$	$>99 \pm 0$	$<1 \pm 0$
PQ (1 mM)	0	$>99 \pm 0$	60 ± 3	33 ± 2*	60 ± 2*	16 ± 2*
Glucose (54 mM)	0	100	2 ± 1	0	$>99 \pm 0$	3 ± 1
Glucose(54 mM) + PQ (1 mM)	0	$>99 \pm 0$	61 ± 2*	2 ± 1	98 ± 1	11 ± 2*
Vit C (0.5 mM)	0	100	2 ± 1	$<1 \pm 0$	$>99 \pm 0$	0
Vit C(0.5 mM) + PQ (1 mM)	0	$>99 \pm 0$	3 ± 1	3 ± 1	98 ± 1	0
Vit E (0.5 mM)	0	100	0	$<1 \pm 0$	$>99 \pm 0$	0
Vit E (0.5 mM) + PQ (1 mM)	0	$>99 \pm 0$	2 ± 1	$<1 \pm 0$	$>99 \pm 0$	0
NAC (1 mM)	0	100	0	0	100	0
NAC (1 mM) + PQ (1 mM)	0	$>99 \pm 0$	2 ± 1	$<1 \pm 0$	$>99 \pm 0$	0
DES (100 nM)	0	$>99 \pm 0$	0	$<1 \pm 0$	$>99 \pm 0$	0
DES (100 nM) + PQ (1 mM)	0	$>99 \pm 0$	0	$<1 \pm 0$	$>99 \pm 0$	0
CP55,940 (100 nM)	0	100	0	0	100	0
CP55,940 (100 nM) + PQ (1 mM)	0	$>99 \pm 0$	0	$<1 \pm 0$	$>99 \pm 0$	0
JWH-015 (100 nM)	0	100	0	0	100	0
JWH-015 (100 nM) + PQ (1 mM)	0	$>99 \pm 0$	0	$<1 \pm 0$	$>99 \pm 0$	0
IGF-1 (100 nM)	0	100	0	$<1 \pm 0$	$>99 \pm 0$	$<1 \pm 0$
IGF-1 (100 nM) + PQ (1 mM)	0	$>99 \pm 0$	60 ± 2	$<1 \pm 0$	$>99 \pm 0$	19 ± 2*

Lymphocytes cells were incubated with PQ (1 mM) in absence or presence of glucose (54 mM), Vit C (0.5 mM), Vit E (0.5 mM), NAC (1 mM), DES (100 nM), CP55,940 (100 nM), JWH-O15 (100 nM), and IGF-1 (100 nM) for 24 h. Evaluation of superoxide anion radicals, mitochondrial depolarization and apoptosis was performed after 1 h of incubation as described in *Materials and methods*. Quantification of superoxide anion radicals (formazan production), mitochondrial depolarization (fluorescent green cells $D_iOc_6(3)$ and apoptosis is expressed as mean of percentage (%) + SD from three independent experiments. *$p < 0.05$ vs. respective control as determined by the Student's *t*-test.

(Fahn and Cohen 1992), but whether it plays a causative role or is a consequence of the pathogenic sequence of the neurodegenerative process is still controversial. The herbicide PQ has been used as classical xenobiotic compound to model PD (Bove et al. 2005). Although, it is widely accepted that PQ generates ROS such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$)-provided that iron ions are available in the aqueous milieu-, its detailed toxic action(s) at a single cell level has not yet been fully described. In the present study, we provide *in vitro* evidence supporting a causative role for oxidative stress in PQ-induced death in lymphocytes cells in a domino-like mechanism

Table III. Hydroxyl radicals are involved in PQ toxic effect on lymphocytes.

Treatment/assay	AO/EB (%)	$D_iOc_6(3)$ (%)
Untreated	$<1 \pm 0$	$>99 \pm 0$
PQ (1 mM)	33 ± 2*	60 ± 2*
Fe^{2+} (0.025 mM)	$<1 \pm 0$	$>99 \pm 0$
H_20_2 (0.1 mM)	47 ± 3	37 ± 3
H_20_2 (0.1 mM) + Fe^{2+}	72 ± 3	37 ± 3
PQ (1 mM) + Fe^{2+} (0.025 mM)	33 ± 2	62 ± 2
DMSO (0.5 mM)	$<1 \pm 0$	$>99 \pm 0$
DMSO (0.5 mM) + Fe^{2+} (0.025 mM)	$<1 \pm 0$	$>99 \pm 0$
DMSO (0.5 mM) + H_20_2 (0.1 mM)	43 ± 2	44 ± 3
DMSO (0.5 mM) + H_20_2 (0.1 mM) + Fe^{2+} (0.025 mM)	40 ± 3*	57 ± 3*
DMSO (0.5 mM) + PQ (1 mM)	14 ± 3*	84 ± 2*
DMSO (0.5 mM) + PQ (1 mM) + Fe^{2+} (0.025 mM)	16 ± 3*	86 ± 2*
MEL (100 nM)	0	100
MEL (100 nM) + Fe^{2+} (0.025 mM)	$<1 \pm 0$	$>99 \pm 0$
MEL (100 nM) + H_20_2 (0.1 mM)	34 ± 2*	62 ± 2*
MEL (100 nM) + H_20_2 (0.1 mM) + Fe^{2+} (0.025 mM)	51 ± 3*	52 ± 3*
MEL (100 nM) + PQ (1 mM)	$<1 \pm 0$*	$>99 \pm 0$*
MEL (100 nM) + PQ (1 mM) + Fe^{2+} (0.025 mM)	$<1 \pm 0$*	$>99 \pm 0$*

Lymphocytes cells were incubated with PQ (1 mM) in absence or presence of Fe^{2+} (0.025 mM), H_2O_2 (0.1 mM), DMSO (0.5 mM) and MEL (100 nM), or in combination as indicated for 24 h. Evaluation of apoptosis and mitochondrial depolarization were performed by AO/EB staining and $D_iOc_6(3)$ fluorescent dye as described in *Materials and methods*. H_2O_2 (0.1 mM) plus Fe^{2+} (0.025 mM) were used as positive control. Quantification of apoptosis and mitochondrial depolarization is expressed as mean of percentage (%) + SD from three independent experiments. *$p < 0.05$ vs. respective control as determined by the Student's *t*-test.

Table IV. Natural or artificial antioxidant compounds, IGF-1 and glucose rescue experiments against PQ.

Treatment/assay	AO/EB (%)
Untreated	$<1 \pm 0$
PQ (1 mM) + medium	$33 \pm 2^{\star}$
Glucose (54 mM) + PQ (1 mM)	5 ± 2
Vit C (0.5 mM) + PQ (1 mM)	7 ± 2
Vit E (0.5 mM) + PQ (1 mM)	$<1 \pm 0$
NAC (1 mM) + PQ (1 mM)	1 ± 1
CP55,940 (100 nM) + PQ (1 mM)	2 ± 1
JWH-015 (100 nM) + PQ (1 mM)	2 ± 1
IGF-1 (100 nM) + PQ (1 mM)	2 ± 1
MEL (100 nM) + PQ (1 mM)	2 ± 1
PDTC (10 nM) + PQ (1 mM)	4 ± 1
PFT (50 nM) + PQ (1 mM)	3 ± 1
DEVD (10 mM) + PQ (1 mM)	2 ± 1

Lymphocytes cells were exposed to listed products at 6 h of 1 mM PQ post-treatment. Quantification of apoptosis is expressed as mean of percentage (%) + SD from three independent experiments. $^{\star}p < 0.05$ vs. respective control as determined by Student's *t*-test.

involving $O_2^{\cdot-}/H_2O_2/^{\cdot}OH$ generation (Figure 2(A),(B), Table III), mitochondrial damage (Figure 2(B)), transcription factor activation (Table I, Figure 3) converging in caspase-3 activation (Table I), and apoptosis (Figure 1). Moreover, using either natural or artificial antioxidant compounds (Tables II and III), we demonstrate that $O_2^{\cdot-}/H_2O_2$ production is essential in PQ-induced cytotoxicity. Most importantly, by using antioxidant compounds and pharmacological signaling inhibitors, it is shown that lymphocytes were rescued from PQ-induced apoptosis (Table IV). Specifically, lymphocytes displayed the typical apoptotic morphology features such as chromatin condensation and/or nuclei fragmentation as early as 6 h post-PQ (1 mM) treatment. This result suggests that PQ provokes apoptosis in a time- and dose dependent fashion. As for lymphocytes, PQ targets nigrostriatal dopaminergic neurons (McCormack et al. 2002), astrocytes (Schmuck et al. 2002), microglia (Miller et al. 2007), cerebellar granule cells (Gonzalez-Polo et al. 2004), cortical cells (Kim et al. 2004), and SHSY-5Y cell line (McCarthy et al. 2004), yet, its mode of cell entry is not yet fully understood. While some investigators (Shimizu et al. 2001, 2003) have involved the dopamine transporter (DAT) in PQ transport on organotypic midbrain culture, others have either found that the herbicide has not effect on dopamine uptake in striatal synaptosomes *in vitro* (Barlow et al. 2003) or that PQ is neither a substrate nor inhibitor of DAT (Richardson et al. 2005). The reason of these contradictory results is at present unknown, but differences in tissue, cell type and/or technical approaches might be a reasonable explanation. Strikingly, we find that the biogenic amine transport inhibitor DES completely blocked PQ-induced apoptosis in lymphocytes to control values ($<1\%$ AO/EB staining). This observation suggests that monoaminergic (i.e. serotonin, dopamine and noradrenalin) reuptake system, which is present in lymphocytes (Marino et al. 1999) as well as in dopaminergic neurons, may be implicated in PQ-neurotoxicity.

It is generally accepted that PQ produces ROS. Effectively, by using the reduction of NBT into formazan, oxidation of DHR into fluorescent rhodamine assay, and the $^{\cdot}OH$ scavengers DMSO and MEL, we confirm that PQ produced $O_2^{\cdot-}/H_2O_2/^{\cdot}OH$ in a time-dependent manner (Bonneh-Barkay et al. 2005). However, the staining pattern displayed by NBT, i.e. while dot-like blue–purple precipitates were observed in the cellular cytoplasm at 1 h of PQ exposure, a homogenous cytoplasmic blue–purple precipitates surrounding the nucleus was detected at 24 h of PQ exposure, suggests that either $O_2^{\cdot-}$ radicals are generated in the cytoplasm (Gray et al. 2007) or at the mitochondria (Fukushima et al. 1993; Castello et al. 2007). Consequently, at present, the role of NADPH/NADH reductases, complex I (NADH: ubiquinone oxidoreductase) or complex III (ubiquinol: cytochrome *c* oxidoreductase) in the PQ monocation radical (PQ^{+}) production remains to be established. Despite these uncertainties, our data suggest that the ultimate target of PQ cycling damage might be the mitochondria. Moreover, we evidence that mitochondria play an important role in the pro-apoptotic signaling pathway triggered by PQ in lymphocytes. This conclusion is supported by the following observations. First, time course of mitochondrial transmembrane potential ($\Delta\Psi_m$), assessed by $D_iOc_6(3)$ assay, clearly showed impairment of mitochondrial function as early as 6 h. This result is in agreement with the notion that the early stage of the apoptotic processes is characterized by a substantial reduction in $\Delta\Psi_m$ that precedes the later stages of nuclear disintegration. Second, it is known that PQ -in the millimolar range- depletes intracellular NADH and ATP in hepatocytes (Palmeira et al. 1994), and that glucose metabolism is the key point in preserving the $\Delta\Psi_m$ and maintaining the balance between life and death (Moley and Mueckler 2000). Thus, we determine the $\Delta\Psi_m$ at 1 h and at 24 h post-PQ treatment in the presence of glucose (54 mM) and demonstrate that, regardless of $O_2^{\cdot-}$ production, the percentage of $\Delta\Psi_m$ at any interval of time is not impaired by PQ when compared to untreated cells or treated with glucose alone, thereby affording lymphocytes complete protection from apoptosis (Table II). In addition, the extent of $\Delta\Psi_m$ reduction over time in PBL cells not only correlated well with impairment in their function, as assessed by $D_iOc_6(3)$, but also correlated well with their apoptotic morphology after PQ exposure, as assessed by a conventional AO/EB staining technique (Figure 2(B)). These observations suggest that change in $\Delta\Psi_m$ is involved in the apoptotic process and that glucose possible serves as an important regulatory factor in the PQ-induced

toxic cascade. In keeping with this, it is relevant to point out that the mitochondrial outer membrane permeabilization (MOMP) is considered the "point-of-no-return" of cell demise as this event is responsible for engaging the apoptotic cascade in numerous cell death pathways. Interestingly, MOMP can originate from either mitochondria outer membrane (MOM) or inner membrane (MIM), and depending on the stimuli and/or cellular context, loss of the $\Delta\Psi_m$ can occur before, during or after MOMP (Armstrong 2006). Taken this information and our findings suggest that the use of glucose as a source of energy (e.g. ATP) is essential to preserve the $\Delta\Psi_m$ as a decisive step in the resistance against oxidative stress caused by PQ. Moreover, low percentage of $D_iOc_6(3)$ in PQ-treated cells when compared to glucose plus PQ suggest that loss of $\Delta\Psi_m$ might occur before MOMP. Interestingly, by using the specific caspase-3 inhibitor Ac-DEVD-cho, it is shown that lymphocytes were completely protected and/or rescue against the noxious effects of PQ (Tables II and IV). Although, the role of mitochondria in PQ cell death signaling is unquestionably, our data indicate that MOMP does not the "point-of-no-return" in PQ-induced apoptosis but caspase-3 activation might be the commitment--to-die in lymphocytes. Third, kinetically evaluation of H_2O_2 production by PQ and bonus addition of H_2O_2 (100 μM) to lymphocytes provoked a profound alteration in the $\Delta\Psi_m$ concurrently with apoptosis (Table III). This observation is in accordance with the idea that H_2O_2 precedes loss of $\Delta\Psi_m$ (Tada-Oikawa et al. 1999). Taken together, our data suggest that mitochondrion is central in PQ evoked cell demise, however, it is possible to rescue cells from death signalling by pharmacological caspases inhibition (Lavrik et al. 2005). Fourth, glucose was able to maintain the $\Delta\Psi_m$ functionally and nuclear morphology against PQ toxicity to control values (100% $D_iOc_6(3)$ fluorescent staining and $<1\%$ AO/EB nuclear staining, Table II). Accordingly, it has been reported that either pyruvate or sodium pyruvate, a natural metabolic intermediate and energy substrate, inhibited the collapse of $\Delta\Psi_m$ and apoptosis from H_2O_2-induced oxidative stress in human neuroblastoma SK-N-SH (Wang et al. 2007) and SK-N-MC cell line (Jagtap et al. 2003). Finally, natural or synthetic antioxidants and hydroxyl scavengers were able to inhibit the reduction of NBT, thereby maintaining the $\Delta\Psi_m$ functionally and nuclear morphology of cells when co-incubated with PQ (Tables II and III). Taken together, our findings suggest that lymphocytes under PQ exposure, the mitochondria de-energize and depolarize as a consequent of ROS, leading to apoptotic cell death.

It has been demonstrated that PQ induces dopaminergic nigral apoptosis in cell lines as well as in mouse brain by activating JNK and c-Jun (Peng et al. 2004; Niso-Santano et al. 2006; Klintworth et al. 2007). In this work, we confirm that PQ activated c-Jun (Table I, Figure 3(F)). Amazingly, NF-κB transcription factor is also involved in PQ death signaling, as assessed by immunohistochemical staining (Figure 3(D)) and pharmacological inhibition by PDTC (Table I). As for other well characterized redox cycling neurotoxin which are producers of H_2O_2 (e.g. 6-OHDA, 5,6-DHT) and transcription factor activators (Jimenez-Del-Rio and Velez-Pardo 2002), these observations suggest that NF-κB and JNK signaling might be activated simultaneously under PQ/H_2O_2-induced oxidative stress in lymphocytes. Furthermore, this is the first report an *in vitro* study to establish a link between NF-κB and c-Jun activation to be involved in PQ/H_2O_2-induced apoptosis signaling. Although it has been suggested that NF-κB activation by H_2O_2 is highly cell-type specific and involves different mechanisms, H_2O_2 has been shown to induce activation of NF-κB in neuronal cells such as cerebellar granule cells (Kaltschmidt et al. 2002) as well as in non-neuronal cells such as HeLa cells (Kaltschmidt et al. 2000); endothelial cells (Aoki et al. 2001); CEM-C7 T-cells, and Jurkat T leukaemia cells (Dumont et al. 1999), intestinal epithelial cells (Li et al. 2002) and human lymphocytes (Velez-Pardo et al 2002) probably via a common mechanism which involves the activation of the so called IκB-kinase (IKK) complex by the SH2-containing Inositol 5-phospatase 1 (SHIP-1) pathway (Gloire et al. 2006). Once activated by phosphorylation, the IKK complex phosphorylates IκBα on Ser32 and Ser36, which is subsequently ubiquitinated and degraded via proteosome system. The freed NF-κB then translocates into the nucleus where it activates the transcription of pro-apoptotic target genes such as p53. In fact, immunohistochemical detection of p53 (Figure 3(E)) may indicate that p53 can be directly up-regulated by NF-κB. Moreover, pharmacological inhibition of p53 by PFT completely abolishes PQ evoked apoptosis (Table I) complying with the notion that p53 is involved in the mechanism of PQ toxicity (Takeyama et al. 2004). It is noteworthy to mention that p53 is able to transcribe pro-apoptotic genes such as Bax (Miyashita and Reed 1995), which in turn, is able to permeabilize the mitochondria promoting the release of the apoptogenic cytochrome *c* (Heimlich et al. 2004), which in turn leads to caspase-3 activation and apoptotic morphology (Figure 1(A), Table I). Similar to NF-κB, H_2O_2 is capable to induce activation of JNK/c-Jun (Kim et al. 2000). How, then, these concurrent signaling pathways are activated by PQ/H_2O_2? One possibility is that once activated the mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1), it may serve as a cross talk molecule between the JNK and NF-κB pathway. Indeed, MEKK1 has been shown to mediate phosphorylation of the IκBα (i.e. the repressor of NF-κB) by IKK and MKK4/MAPK kinase. Noticeably, MKK4

phosphorylates JNK/SAPK, which phosphorylates c-Jun transcription factor. Thus, MEKK1 is a critical component of both the c-Jun and NF-κB stress response pathways (Jimenez-Del-Rio and Velez-Pardo 2004 and references within). Taken together our findings, it is not surprising to assume that NF-κB and JNK/c-Jun are accomplices with each other during PQ exposure in lymphocytes. Despite these advances, the current significance of NF-κB/c-Jun activation induced by oxidative stress in the human brain is awaiting further investigation.

Previous studies have demonstrated that antioxidants such as vitamin C (ascorbic acid), vitamin E (α-Tocopherol), and NAC were highly effective in the treatment of PQ toxicity mainly due to its chemical capability to scavenge/quench free radicals (Suntres 2002). In fact, these three antioxidant compounds were able to blunt the production of oxygen radicals by interfering with the PQ cycling (i.e. from PQ^{2+} to PQ^{+}). Furthermore, our data suggest that antioxidants confers protection either by keeping PQ up in its oxidized form (i.e. PQ^{2+}) or by reducing superoxide radicals (Table II). This notion is further reinforced by the fact that JWH-015 and CP55,940, two cannabinoid antioxidants, completely protect lymphocytes against PQ-superoxide radicals (Table II) and H_2O_2 (Velez-Pardo and Jimenez-Del-Rio 2006) toxicity. These results imply that pretreatment of PBL cells with antioxidants may confers protection against PQ deleterious effects. We report for the first time that IGF-1, a growth factor, is able to confer lymphocytes complete protection against PQ probably via receptor-dependent mechanism involving p53 turning-down (Jimenez-Del-Rio and Velez-Pardo 2006). Moreover, we also report for the first time that natural (e.g. vitamin C, E; MEL) or artificial (e.g. NAC, JWH-015, CP55,940) antioxidants, IGF-1, glucose and specific pharmacological inhibitors (e.g. PDTC, PFT, Ac-DEVD-cho) of NF-κB, p53 and caspase-3 were capable to rescue lymphocytes from apoptotic cell death pre-exposed to PQ (Table IV). Interestingly, it has been reported that sodium salicylate, a well established $^{\cdot}OH$ scavenger and NF-κB inhibitor, strongly suppressed PQ-induced lung toxicity, and this effect was associated with 100% survival of PQ-treated rats (Dinis-Oliveira et al. 2007). Taken together, these observations and our results suggest that there are different compounds with varied chemical properties and mechanisms of action able to abolish and rescue lymphocytes from the toxic effect of PQ.

In summary, we provide evidence that PQ induces apoptosis in lymphocytes by a sequential cascade of molecular events involving the production of superoxide radicals, hydrogen peroxide and hydroxyl radicals which in turn trigger a specific cell death pathway characterized by the simultaneous activation of NF-κB/p53/c-Jun transcription factors, mitochondrial depolarization and caspase-3 activation pathway (Figure 4). We also provide evidence that dying lymphocytes can be rescued from PQ noxious stimuli by different mechanisms (e.g. direct antioxidant effect, receptor mediated signaling, energetic protection). These findings may explain why treatment with dextrose (glucose), desferrioxamine, vitamin E and NAC have been successful in a survival case

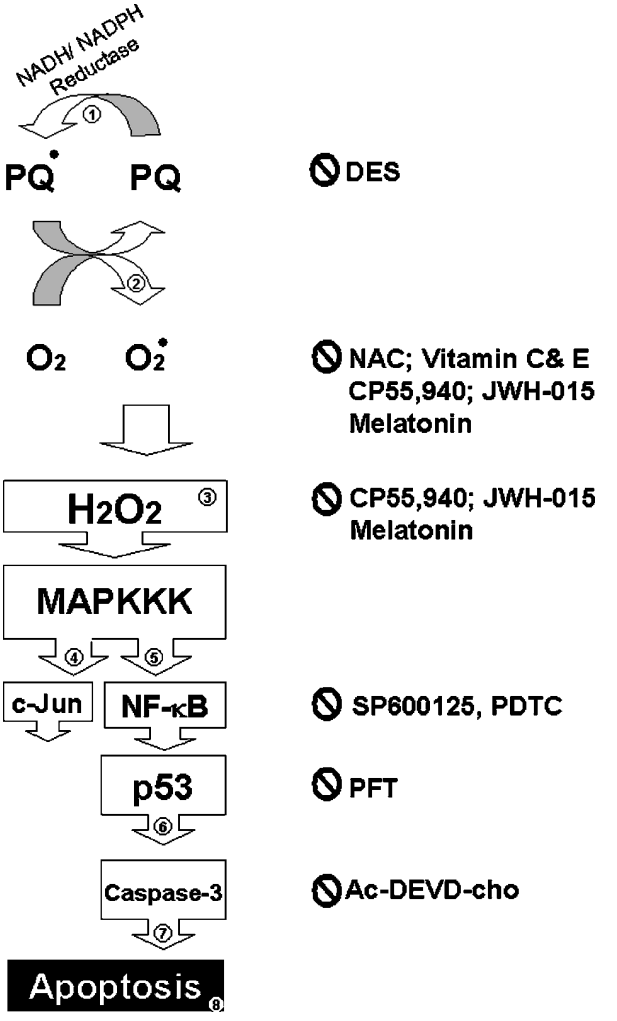

Figure 4. Schematic model of the major molecular events induced by PQ in lymphocytes. PQ in the presence of NADH/NADPH reductases (1) is converted into monocationic radical compound which readily react with molecular dioxygen to generate superoxide radicals (2), which dismutase either by enzymatic (e.g. superoxide dismutase, SOD) or spontaneously into H_2O_2 (3). This last compound in turn may activate the mitogen-activated protein kinase kinase kinase (e.g. MEKK1) which can activate both c-Jun (4) via activation of MKK4/JNK, and NF-κB activation (5) via phosphorylation of the IκBα (i.e. the repressor of NF-κB) by the IKK complex. The NF-κB translocates into the nucleus and transcribes p53 protein (6). Consequently, this protein transcribes pro-apoptotic proteins (e.g. Bax) which are able to permeabilize mitochondria, thus, promoting the activation of caspase-3 (7) which signals chromatin fragmentation, typical of apoptotic morphology (8). The symbol (·) represents the inhibition (by indicated compound) of the critical step of the molecular cascade leading to apoptosis by PQ.

of acute PQ poisoning (Dinis-Oliveira et al. 2006a,b) and vitamin E may have neuroprotective effect attenuating the risk of PD (Etminan et al. 2005). Taken together, our results support the notion that oxidative stress may play an important role in the etiology of PD. Additionally, the present data may contribute to understanding the cell degeneration process in PD, and provide new therapeutic strategies (e.g. cannabinoids, Lastres-Becker and Fernandez-Ruiz 2006, and growth factors) to intervene environmentally and/or genetically susceptible population to oxidative stress as encounter in Antioquia, Colombia (Jimenez-Del-Rio et al. 2004).

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