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Pronounced activation of protein kinase C, ornithine decarboxylase and c-*jun* proto-oncogene by paraquat-generated active oxygen species in WI-38 human lung cells

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Abstract

Paraquat (methyl viologen, PQ) is a widely used herbicide that produces oxygen-derived free radicals and severely injures human lungs. In this study we examined the effects of PQ on the protein kinase C (PKC), ornithine decarboxylase (ODC) and c-*jun* oncogene expression in WI-38 human lung cells. Exposure of cells to 25–200 μM PQ resulted in an increase of [^{3}H]phorbol dibutyrate (PDBu) binding and PKC redistribution in a dose-dependent manner. Interestingly, a superoxide dismutase mimic, 4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, 2.5 mM) and catalase (400 μg/ml) could significantly reduce the PQ-stimulated increase of phorbol ester binding and particular PKC phosphorylating activity, but dimethylsulfoxide (DMSO, 1.5%), an effective ·OH trapping agent, failed to prevent this stimulation. In addition, an endogenous substrate of PKC, 80 kDa protein, was found to be highly phosphorylated in intact WI-38 cells treated with 50 μM PQ. The increase of phosphorylated proteins could be completely or partly abolished by Tempol or catalase, but only the phosphorylation of 80 kDa protein was diminished by protein kinase C inhibitor, 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine (H-7). A maximal peak of ODC activity was observed at 6 h of treatment with 50 μM PQ. PQ induced activity was reduced at the following rates, Tempol 85%, DMSO 80% and catalase 45%, but H-7 failed to do so. Furthermore, we found that the level of c-*jun* mRNA was transiently increased by PQ and the peak appeared at 1 h of treatment. When correlated with the PKC result, Tempol, catalase and H-7 all effectively blocked PQ-elicited c-*jun* transcript expression, but DMSO only exhibited a weakly inhibitory effect. We therefore propose that superoxide anion (O_2^- and H_2O_2 generated by PQ could activate PKC and lead to induction of c-*jun* gene expression; on the other hand, O_2^- and ·OH might trigger other kinase pathways to elevate ODC activity. Finally, the sequential expression of c-*jun* oncogene and ODC may cooperate to relieve the oxidative damages elicited by PQ.

Keywords: Protein kinase C; Ornithine decarboxylase; Oncogene expression; Paraquat

1. Introduction

Paraquat (PQ; N,N′-methyl-4,4′-bipyridyl) is a widely used herbicide which injures the lungs of humans and animals [1,2]. It has been demonstrated that PQ undergoes a one-electron-reduction by the flavoenzyme NADPH-cytochrome p450 reductase [3–7]. The resulting free radicals react rapidily with molecular oxygen to form superoxides with the regeneration of the PQ di-cations. Thus, if the reducing equivalents are sufficient, repeated cycles of PQ reduction and reoxidation will make a mass of reactive oxygen species. The balance between the generation of oxygen radicals and their dissipation by cellular defense systems is therefore disturbed, allowing reactive oxygen species to attack biomolecules. Membrane damage induced by lipid peroxidation, inactivation of proteins or damage to DNA may subsequently lead to cell death. Although the toxic outcomes induced by PQ are being studied extensively, the signaling mechanisms by which PQ transduces the toxic effect to cellular targets remain unclear.

Protein kinase C (PKC) is an important signal of transducer processes including tumor promotion [8], cell differentiation and proliferation [9]. Its activation is the crucial event in the transmembrane signalling of numerous extra-

Abbreviations: PQ, paraquat; PKC, protein kinase C; PDBu, phorbol dibutyrate; Tempol, 4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl; H-7, 1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine; ODC, ornithine decarboxylase; AOS, active oxygen species; DMSO, dimethylsulfoxide.

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cellular stimuli [10]. Recently, it has been demonstrated that active oxygen species could change the subcellular localization and kinase activity of PKC in JB6 mouse epidermal cells [11]. Moreover, the topical application of benzoyl peroxide on mouse skin was found to alter the Ca^{+2} dependency of PKC in basal epidermal cells [12]. Another study showed that exposure of cells or purified PKC to high concentrations of hydrogen peroxide (5 mM) resulted in rapid inactivation of both phorbol ester binding as well as phospho transfer capacity [13].

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines [14], and has been shown to be stimulated in the lungs in response to hyperoxia or PQ [15,16]. It has been suggested that the lung polyamine system in PQ or O_2 toxicity might play a role in repair through scavenging of oxygen free radicals [17]. Recently, ODC was found to act as a proto-oncogene central to regulation of cell growth and transformation [18].

The c-*jun* proto-oncogene belongs to a class of proto-oncogenes which code for nuclear proteins that regulate various aspects of cell growth and differentiation. As an immediate early response gene, expression of the c-*jun* gene is inducible by serum, active phorbol esters, ionizing radiation and oxygen free radicals [19–21]. Several sets of genes participating in cellular protective function, such as metallothioneins [22] and GST-π [23], are preferentially regulated by AP-1 complex, for which the major component is encoded by c-*jun*.

As PQ can be metabolized to generate a large amount of oxygen free radicals when entering cells, we propose that the alteration of the oxidation/reduction status within cells by PQ might modulate 'cellular sensors', thereby leading to transduction of the toxic signals from membrane to the nucleus. This, in turn, may trigger defense systems in response to PQ-generated toxic stress. Based on this hypothesis, we investigated the expression of PKC, ODC, and c-*jun* to determine whether they are affected by treatment with PQ. A plausible sequence of signaling events among these targets in response to PQ is also studied.

2. Materials and methods

2.1. Chemicals

Phenylmethylsulfonyl fluuride (PMSF), leupetin, histone H1(type III-s), phosphatidylserine, 1,2-diolein (1,2-di-oleoly-rac-glycerol), TPA, paraquat, 4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), catalase and H-7 were purchased from Sigma, USA; [γ-^{32}P]ATP (5000 Ci/mmol), [^{3}H]PDBu (20 Ci/mmol), [α-^{32}P]dCTP and [^{14}C-1]ornithine were from Amersam, USA.

2.2. Cell cultures

WI-38 human lung cells were obtained from American Type Culture Collection (ATCC), WI-38 cells were grown in basal Eagle's medium (Gibco) supplemented with 10% fetal calf serum, antibiotics (penicillin at 100 units/ml and streptomycin at 50 μg/ml) and 2 mM glutamine.

2.3. [^{3}H]PDBu binding to intact cells

The binding study was conducted as described in our previous study [24]. Cells were grown on 35-mm plates, and all studies were conducted on confluent populations of cells (roughly 5×10^5 cells/plate), approximately 2 days after subculture. All binding assays were conducted at 37°C in a total volume of 500 μl and were begun by replacing the cell medium with fresh BME. Cells were then exposed either to indicated concentrations of paraquat for 30 min followed by [^{3}H]PDBu (10 nM routinely, or as indicated in legends for Scatchard analysis) for another 10 min, or exposed to fixed doses of paraquat for various times before [^{3}H]PDBu incubation. All assays were terminated by aspirating the binding mixture, rinsing the plates with cold PBS for 3 times, and solubilizing the cells with 0.5 ml of 1 N NaOH. The samples were neutralized with 0.5 ml of 1 N HCl and assessed for tritium content in 3 ml Hydrofluor. Specific binding represents the difference between total binding and that measured in the presence of 10 μM unlabeled TPA.

2.4. Preparation of cytosolic and particulate fractions of PKC

Cytosolic and particulate associated PKC fractions were prepared as previously described [25]. Confluent WI-38 cells ($1–2 \times 10^7$ cells) were incubated for different times in the indicated treatment of paraquat or other test compounds. Cells attached to the culture dish were then washed twice with PBS (0.125 M NaCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4 [pH 7.2]) and twice with buffer A (20 mM Tris [pH 7.5], 2 mM EDTA, 0.5 mM EGTA, 0.3 M sucrose, 2 mM PMSF, 10 μg/ml leupeptin). The cells were scraped into 10 ml of buffer A and homogenizied with 30 strokes. The homogenate was centrifuged at $100\,000 \times g$ for 1 h at 4°C. The supernatant was stored at −70°C for cyotsolic fraction, and the pellet was washed with buffer B (20 mM Tris [pH 7.5], 2 mM EDTA, 2 mM PMSF) by centrifugation (15 min, $12\,000 \times g$). The pellet was resuspended in 10 ml of buffer A and homogenizied with 30 strokes, as described above. Triton X-100 was added to the particulate suspension at a final concentration of 0.1%, and incubated for 1 h at 4°C in a rotating mixer. Centrifugation (1 h, $100\,000 \times g$) was used to remove any nonsolubilized material, and the supernatant then was store at −70°C as particulate fraction.

2.5. Protein kinase C activity measurement

Protein kinase C activity was assayed by counting the transfer of γ-^{32}P-labeled phosphate from ATP to histone,

as described elsewhere [26,27]. The incubation mixture (0.2 ml) contained 5 μmol Tris-HCl (pH 7.4), 2 μmol $MgCl_2$, 40 μg of lysine-rich histone, 10 μg of phosphatidylserine, 0.75 μg of 1,2-diolein, 10 μg leupeptin, 0.35 μmol $CaCl_2$, 0.12 μmol EGTA and 400 fmol of [γ-^{32}P]ATP containing 350000 cpm. Blank samples were incubated in the absence of phosphatidylserine and diolein. The reaction was carried out at 30°C for 3 min, and terminated by addition of 1 ml of cold 20% (w/v) TCA, and the phopshorylated histone was precipitated by filtration of the sample on the Millipore HA 0.45-μm filters, rinsing the filters with 2 ml 5% TCA. The tubes were washed with 1 ml of cold 20% TCA and applied to the filters. The filters were then washed 5 times with 3 ml of cold 5% TCA and counted. The PKC activity was expressed as *p*mol of ^{32}P incorporated per 3 min per mg protein of partial purified sample.

2.6. Determination of protein phophorylation

WI-38 cells were cultured and labelled in vivo as previously described [24]. Briefly, cells were labelled with 0.2 mCi of [^{32}P]Pi in 2 ml of phosphate-free BME. Subsequently, cells were treated with various doses of paraquat for 30 min or a fixed dose for different times. After that, cells were washed twice in ice-cold Tris-buffer saline (0.15 M NaCl, 20 mM Tris [pH 7.5]), then extracted twice with 5% TCA, rinsed with Tris-buffered saline twice, and finally lysed in 250 μl of boiling SDS sample buffer. The samples were then boiled for 5 min, centrifuged briefly, and equal amount of protein (500 μg) in each sample was electrophoresed on 10% SDS-polyacrylamide gels. After drying gels were autoradiographed at −80°C, using Kodak X-ray film.

2.7. ODC activity assay

Measurement of ODC activity was performed as previously described [28]. Briefly, cells were grown to confluence in 100 × 20 mm culture dishes. Cell monolayers were rinsed and cultured for an additional 12 h in serum-free media prior to treatment with PQ or other reagents. After incubation (as indicated) cell monolayers were rinsed three times with ice-cold phosphate-buffered saline, collected by scraping with a rubber policeman, and pelleted by centrifugation (300 × *g* for 5 min) at 4°C. The cell pellet was sonicated on ice in 0.6 ml of ODC assay buffer (50 mM Na-K phosphate [pH 7.2], 0.1 mM EDTA, 1.0 mM dithiothreitol, 40 μM pyridoxal phosphate) and following centrifugation (12000 × *g* for 15 min), the supernatant was assayed in triplicate for ODC activity. Samples were assayed in a total volume of 0.2 ml containing 0.25 μCi L-[^{14}C-1]ornithine. Assays were incubated 60 min at 37°C and reactions were terminated by the addition of 0.5 ml of 2 M citric acid injected directly into the reaction mixture. Activity was measured by trapping the evolved $^{14}CO_2$ in 0.2 ml hyamine hydroxide, which was suspended in a well above the assay mixture. Trapped $^{14}CO_2$ was counted by liquid scintillation and activity is defined in units of ODC (nmol CO_2 evolved/h/mg protein). Protein was determined by Bio-Rad protein assay.

2.8. RNA isolation and analysis

A full-length c-*jun* coding sequence [29] and a GAPDH cDNA *pst*I fragment [30] cut from pIBI130GAPDH plasmid were random-primer labeled and used as probes in the Northern blot analysis. Total RNA from the cells grown in 100-mm petri dishes was isolated by 4 M guanidine isothiocyanate [31]. Fifteen μg of RNA were used for Northern blotting as previously described [32].

3. Results

3.1. Effects of PQ on PKC

The binding assay of the phorbol ester derivative PDBu to intact cells was used to measure the ligand binding capacity of cell surface protein kinase C. In a time course experiment, a rapid binding stimulation was observed after 15 min of treatment with 50 μM PQ, and reached a maximal binding at 30 min (data not shown). Table 1 shows that exposure of cells to 25–200 μM PQ for 30 min resulted in a dose-dependent increase of radioactive PDBu binding. To investigate whether the stimulation of [^{3}H]PDBu binding by PQ was due to the generation of active oxygen species (AOS), several radical savengers were examined for their abilities to prevent this stimulation. As shown in Table 1, treatment of cells with catalase (400 μg/ml) and Tempol (2.5 mM) resulted in a 34% and 55% inhibition of [^{3}H]PDBu binding, respectively, as compared to paraquat-treated cells. In contrast, DMSO

Table 1
Effect of PQ on [^{3}H]PDBu binding to intact WI-38 cells

Treatment [a]	Bound [^{3}H]PDBu cpm/10^6 cells	% of inhibition [b]
None (control)	2015 ± 85	–
PQ (25 μM)	2930 ± 121	–
PQ (50 μM)	4112 ± 180	–
PQ (100 μM)	4210 ± 215	–
PQ (200 μM)	4332 ± 122	–
PQ (50 μM) + Tempol (2.5 mM)	2965 ± 113	55
PQ (50 μM) + catalase(400 μ/ml)	3300 ± 132	34
PQ (50 μM) + DMSO (1.5%)	4002 ± 187	0

[a] Cells were exposed to [3H]PDBu (10 nM) for 10 min after treatment with PQ or PQ plus inhibitors as indicated above for 30 min. Specific binding was determined as described in Section 2. These values are mean ± S.D. of triplicate samples in a single experiment. Similar results were obtained in two separate experiments.

[b] % of inhibition = 100% − {[cpms of (PQ plus inhibitors − control) group]/[cpms of (PQ − control) group]} × 100.

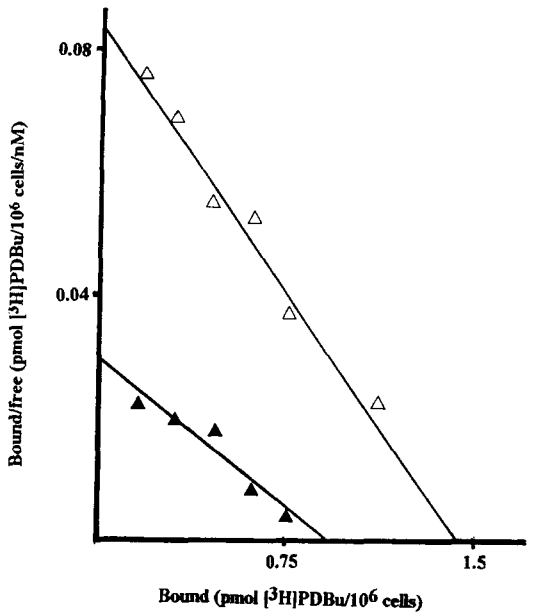

Fig. 1. Paraquat-stimulated [^{3}H]PDBu binding to intact WI-38 cells. Characterization by Scatchard analysis. Cells were cultured as described before and incubated with various concentrations (5–60 nM) of [^{3}H]PDBu for 10 min after addition of 50 μM paraquat or at a corresponding volume of DMSO (control). After an appropriate time of incubation, an aliquot of medium was withdrawn for determination of unbound [^{3}H]PDBu. Dishes were then rinsed with ice-cold buffer, and cells were lysis as described in Section 2. ▲, control; △, paraquat-treated.

(1.5%) failed to inhibit the paraquat-induced [^{3}]PDBu binding in the same cells. However, these radical scavengers had no effect on the radioactive phorbol ester binding by themselves (data not shown).

The nature of the increase of [^{3}H]PDBu binding induced by PQ (50 μM) was further studied by Scatchard plot analysis. Cells were exposed to 50 μM PQ for 30 min and then incubated with increasing concentrations of [^{3}H]PDBu (5–60 nM) for another 10 min. The data in Fig. 1 indicate that the PQ-induced increase of binding is likely to be due to a combination of increased receptor affinity and maximal binding capacity. The dissociation constant for untreated controls was 36 ± 4 nM and for PQ-treated cells it was approximately 24 ± 2 nM. Maximal binding capacity increased from 1.3 ± 0.21 $p$mol/10^6 cells in untreated controls to 2.1 ± 0.33 $p$mol/10^6 cells upon treatment.

The increase of [^{3}H]PDBu binding in intact cells was induced by PQ, suggesting that the activity of protein kinase C might be elevated. To verify this, we isolated the membrane-bound and cytosolic PKC from PQ-treated cells and measured their activity by utilizing histone as a substrate. Table 2 shows that treatment of cells with 25–200 μM of PQ for 30 min induced a redistribution of PKC activity from the cytosol to the membrane in a dose-dependent manner. In the same experiment, we used a typical PKC activator, TPA (0.1 μg/ml) to induce an obvious redistribution of PKC as the positive control. In order to further explore the mechanism of PKC activation by PQ, we concomitantly treated cells with PQ and radical scavengers or H-7 (PKC inhibitor). Subsequent PKC activity of particular fractions was determined. The data in Table 2 show that Tempol (2.5 mM) and catalase (400 μg/ml) caused 82% and 51% of reduction of PKC activity induced by PQ. In contrast, DMSO (1.5%) did not exhibit this inhibition. These results are consistent with those observed in the study of [^{3}HPDBu binding. Moreover, H-7 was found to completely abolish the PQ induced PKC activity.

3.2. Effects of PQ on protein phosphorylation

Active PKC in intact cells can induce a rapid increase of phosphorylation of an 80 kDa protein [33,34]. As shown in Fig. 2, 50 μM PQ induced two phosphoproteins within 30 min with apparent molecular weights of 80 kDa (upper band) and 75 kDa (lower band), respectively. When cells were exposed to TPA (100 ng/ml) for 30 min, an obvious increase of phosphorylation of 80 kDa protein band was also observed, suggesting that this protein might be the specific substrate for PKC. Moreover, it was found that 30 μM H-7 completely eliminated the 80 kDa phosphoprotein band, but failed to reduce the lower one. Interestingly, these two phosphoproteins in the PQ-treated cells were partially reduced by catalase (400 μg/ml) treatment, but both of them were completely eliminated by the addition of Tempol (2.5 mM).

3.3. Induction of ODC activity by PQ

The time-dependent effect of PQ (50 μM) on ODC activity in WI-38 cells is shown in Fig. 3. Six hours after

Table 2
Effect of PQ on PKC activity in WI-38 cells

Treatment [a]	PKC activity (pmol P/mg protein)		% of inhibition [b]
	Cytosol	Particulate	
PQ (μM)			
0	2311±88	731±25	
25	2138±65	955±16	
50	1439±57	1502±34	
100	1186±32	1771±89	
200	1094±25	1202±54	
TPA (100 ng/ml)	1042±77	1820±55	
PQ (50 μM)			
+H-7 (30 μM)		775±21	100
+Tempol (2.5 mM)		984±43	82
+catalase (400 μg/ml)		1176±23	51
+DMSO (1.5%)		1487±67	0.25

[a] Cells were incubated with PQ or PQ plus inhibitors as indicated above for 30 min. Cells were then harvested, and subcellular fractions prepared by DEAE-cellulose chromatography as described in Section 2. PKC activity was determined for cytosolic and particulate fractions. Values represent means ± S.E.M. of 3 separate experiments.
[b] The definition of '% of inhibition' as described in Table 1.

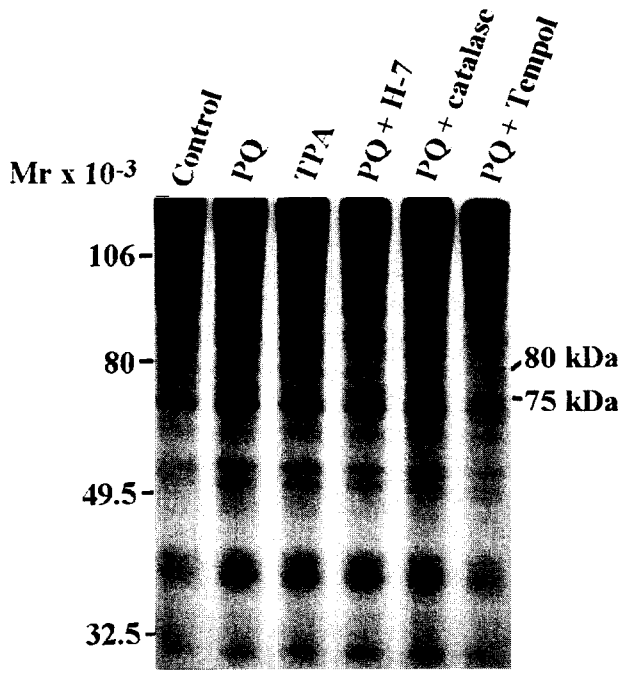

Fig. 2. Effect of paraquat on phosphorylation of cellular proteins in intact WI-38 cells. The detailed procedure is as described in Section 2. The paraquat-stimulated phosphorylation of 80 and 75 kDa are indicated by a mark to the right of the gel, and the positions of molecular weight standards are indicated to the left of the gel. The concentrations of chemicals used here are: paraquat, 50 μM; TPA, 100 ng/ml; H-7, 30 μM; catalase, 400 μg/ml; Tempol, 2.5 mM. Similar results were obtained in three independent experiments.

PQ addition to the cells, a 3- to 4-fold (a maximal peak) induction of ODC activity was detected when compared with untreated cells. ODC activity remained elevated 12–24 h after PQ addition and declined toward control activity 36 h after treatment. The concentration-dependent effect of PQ on ODC activity in WI-38 human lung cells was measured 6 h after addition of PQ to cell cultures (Table 3). Doses as low as 25 μM PQ resulted in changes of

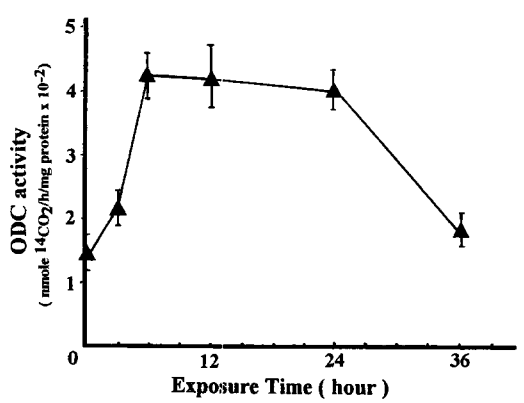

Fig. 3. Time course of PQ effects on ODC activity in WI-38 cells. WI-38 cells were treated with 50 μM of PQ for various times, as indicated above. Cells from triplicate dishes for each time point were prepared and assayed for ODC activity as described in Section 2. Results are reported as the average of triplicate assays of triplicate dishes ± S.E.M.

Table 3
Effect of PQ on ODC activity in WI-38 cells

Treatment [a]	ODC activity nmol^{14} CO_2 / h/mg protein	% of inhibition [b]
PQ (μM)		
0	112 ± 8	
25	220 ± 35	
50	404 ± 39	
100	489 ± 25	
200	542 ± 12	
PQ (50 μM)		
+ Tempol (2.5 mM)	156 ± 19	85
+ DMSO (1.5%)	170 ± 24	80
+ catalase (400 μg/ml)	243 ± 16	45
+ H-7 (30 μM)	398 ± 25	0
+ cycloheximide (10 μg/ml)	101 ± 15	100

[a] Cells were incubated with various concentrations of PQ or a fixed dose of PQ (50 μM) plus inhibitors for 6 h. The detailed procedure for the assay of ODC activity is described in Section 2. Values represent means ± S.E.M. of 3 separate results.
[b] The difinition of '% of inhibition' as shown in Table 1.

basal ODC activity. A dose-dependent induction of ODC activity was observed at the range of 25 to 200 μM. Tempol and catalase were also utilized to determine the role of AOS in PQ-induced ODC activity. As shown in Table 3, Tempol and DMSO effectively abolished PQ-induced ODC activity by 85% and 80%, respectively. Cata-

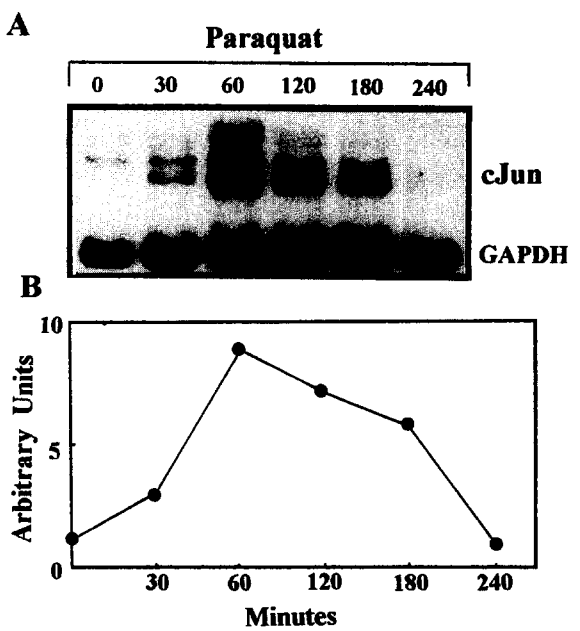

Fig. 4. The kinetics of the expression of c-*jun* by PQ in WI-38 cells. (A) Northern blot analysis of total cellular RNA isolated from WI-38 cells exposed to PQ (50 μM). Cells were harvested at the indicated time points (in minutes), and total cellular RNA was extracted and analyzed on a 1% agarose gel. The blot was hybridized to c-*jun* and GAPDH cDNA probes. (B) Kinetics of c-*jun* expression by PQ. The autoradiogram shown in panel A was quantitated by laser densitometry, and the relative induction of c-*jun* was determined relative to the level of the GAPDH signal.

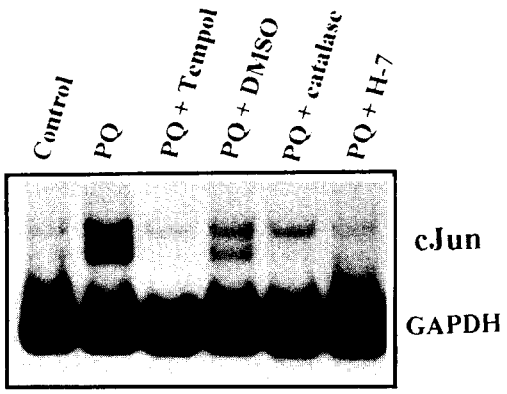

Fig. 5. Effects of antioxidants and PKC inhibitor on c-*jun* expression by PQ. Cultures were incubated with PQ (50 μM) or PQ plus Tempol (2.5 mM), DMSO (1.5%), catalase (400 μg/ml) or H-7 (30 μM) for 60 min. Cells were harvested and total cellular RNA were extracted as described in Fig. 4.

lase only inhibited ODC activity by 45%, while treatment with H-7 (30 μM) failed to inhibit the PQ-induced ODC activity. Additionally, cycloheximide (10 μg/ml) pretreatment of WI-38 cells 1 h prior to PQ addition completely inhibited PQ-induced ODC activity, suggesting that de novo protein synthesis is required for PQ-mediated ODC induction. However, Tempol and DMSO had no effects on the ODC activity by themselves (data not shown).

3.4. Induction of c-jun proto-oncogene expression by PQ

A rapid and transient increase in c-*jun* mRNA was observed after 50 μM PQ treatment in WI-38 human lung cells. The kinetics of the response are shown in Fig. 4B, in which the level of c-*jun* mRNA began to increase within 30 min, peaked at 1 h at a level 8-to 9-fold higher than control, and returned to constitutive levels by 4 h. The level of c-*jun* expression showed a positive dose response to PQ at concentrations between 25 and 200 μM (data not shown), which was consistent with that observed on PKC activity (Table 1 and Table 2). Similarly, Tempol, DMSO and catalase were utilized to investigate whether AOS is involved in PQ-elicited c-*jun* mRNA expression. Our experiments demonstrate that treatment of cells with Tempol or catalase causes a dramatic reduction of the level of c-*jun* transcripts induced by PQ, whereas DMSO caused only partial reduction in the level of PQ induced c-*jun* transcripts (Fig. 5). A remarkable inhibition of PQ-elicited c-*jun* mRNA expression was also observed in the presence of H-7 (30 μM).

4. Discussion

PKC activation represents an important mechanism of signal transduction which participates in the action of numerous biological responses including cellular proliferation and differentiation. It is accomplished by the translocation of the enzyme from the cytoplasm to the plasma membrane and by increasing its kinase activity. Our data show that exposure of WI-38 cells to PQ resulted in an increase of [^{3}H]PDBu binding, membrane-bound PKC activity, and phosphorylation of the PKC substrate 80 kDa protein (Table 1 and Table 2, and Fig. 1 and Fig. 2), suggesting that PQ indeed activated this signal pathway. Simultaneously, we found that the peak of PKC activation rapidly appeared at 30 min after PQ treatment, correlating with the appearance of superoxide anion (O_2^-) (data not shown). Supportive of this finding, Tempol, a metal-independent superoxide dismutase mimic [35], was found to effectively block PQ-induced PKC activation (Table 1 and Table 2, and Fig. 2). In addition to superoxide anion, hydrogen peroxide (H_2O_2) might also be involved in this activation process, as the addition of catalase could partly reduce it. Although TPA has been shown to modulate the production of oxidants in the epidermis [36], its phosphorylated pattern was similar, but not identical, to that of PQ-treated cells (see Fig. 2). The major difference between them was that a 75 kDa protein was highly phosphorylated by treatment with PQ but not with TPA. Moreover, this PQ-elicited 75 kDa phosphoprotein was eliminated by Tempol or catalase, but not by PKC inhibitor, H-7. These observations suggest that PQ not only activates the PKC-mediated signal pathway but also triggers other protein kinase-associated signal mechanisms.

The c-*jun* proto-oncogene is generally known as a stress-responsive gene, because a plethora of stresses, including UV radiation, heat shock and AOS can elevate the expression of this gene, leading to subsequent induction of different subsets of genes, such as metallothionein and GST-π, to relieve such stresses. Our results show that the expression of c-*jun* mRNA was highly induced by PQ in human lung cells. It is interesting to note that addition of H-7, Tempol, or catalase could effectively abolish this induction by PQ, but DMSO alone had little inhibitory effect on it (Fig. 5). We therefore conclude that O_2^- and H_2O_2 had a greater influence on the PQ-induced c-*jun* expression than did hydroxyl radical ($\cdot$OH). These observations concur with those obtained in this study of modulation of PKC activity by three oxygen radical scavengers. Taken together, it might be speculated that PQ-induced c-*jun* expression is mainly caused by PKC-mediated signals, which are initially activated by PQ-elicited O_2^- and H_2O_2. Previous reports have similarly demonstrated that activation of PKC [37] results in rapid induction of the c-*jun* gene concurrently with enhanced AP-1 binding activity.

In contrast to c-*jun*, PQ-stimulated ODC activity was not blocked by addition of H-7, but was inhibited by AOS scavengers. Of the AOS scavengers tested, Tempol and DMSO exhibited a higher inhibitory effect on PQ-induced ODC activity than did catalase, suggesting that O_2^- and $\cdot$OH might play major roles in this activating process.

Thus we concluded that PQ-stimulated ODC activity is not through PKC, but is O_2^- and H_2O_2 dependent. We have suggested (Fig. 3) that PQ, in addition to activating the PKC-mediated pathway, might trigger other kinase-associated signals. Based on these observations, it is reasonable to speculate that the PQ activation of ODC may be through a novel kinase pathway rather than the PKC pathway.

Exposure of mammalian cells to adverse environmental conditions triggers the onset of specific genetic or enzymatic responses, which are likely to have evolved as mechanisms that protect the cell against permanent damage and death. Accordingly, a unique signaling pathway, which is still unknown, could be elicited when PQ insults lung cells. Thus the works identified here partly shed light on the signaling circuitry in response to PQ injury. In conclusion, when PQ enters the cells, it is metabolized to generate large amounts of AOS. Of these, O_2^- and H_2O_2 can activate PKC, leading to induction of c-*jun*. On the other hand, O_2^- and $\cdot OH$ may also stimulate other kinases, in turn elevating the activity of ODC. Finally, the sequential expression of c-*jun* and ODC may interact cooperately to lessen the oxidative stress generated by PQ.

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