Paraquat Induces Apoptosis through Cytochrome C Release and ERK Activation

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Abstract

Paraquat has been suggested to induce apoptosis by generation of reactive oxygen species (ROS). However, little is known about the mechanism of paraquat-induced apoptosis. Here, we demonstrate that extracellular signal-regulated protein kinase (ERK) is required for paraquat-induced apoptosis in NIH3T3 cells. Paraquat treatment resulted in activation of ERK, and U0126, inhibitors of the MEK/ERK signaling pathway, prevented apoptosis. Moreover, paraquat-induced apoptosis was associated with cytochrome C release, which could be prevented by treatment with the MEK inhibitors. Taken together, our findings suggest that ERK activation plays an active role in mediating paraquat-induced apoptosis of NIH3T3 cells.

Key Words: Paraquat, NIH3T3 cells, ERK1/2, Apoptosis, Cytochrome C, MAPK

INTRODUCTION

Paraquat, a redox-cycling compound, is a strong pneumotoxicant (Wallace et al., 2005). Paraquat is also able to induce behavioral and neurological disorders such as Parkinsonism (Mak et al., 2011; Prakash et al., 2013; Qin et al., 2014). The molecular mechanisms responsible for the cytotoxic effects of paraquat in cells are not fully understood but are mainly attributed to their ability to generate of reactive oxygen species (ROS) and subsequent interactions with intracellular macromolecules such as lipids, proteins, and nucleic acids to trigger apoptosis (Rincheval et al., 2012; Chang et al., 2013; Wang et al., 2014c). Recent investigations have shown that paraquat induces apoptosis through mitochondria perturbation including cytochrome C release, subsequent caspase-3 and poly (ADP-ribose) polymerase cleavage (Hong et al., 2013; Han et al., 2014). Paraquat also induced expression of Bcl-2 family proteins such as Bak, Bid, BNip3 and Noxa, and can be triggered apoptosis through extrinsic and intrinsic cell death pathway (Fei and Ethell, 2008; Fei et al., 2008). However, many studies have demonstrated paraquat-induced apoptosis in various cells, but the detailed mechanism of paraquat-induced apoptosis remains largely unknown.

ROS have been shown to participate in the number of human diseases such as cancer, neurodegeneration and aging (Li et al., 2013; Giordano et al., 2014; Kim et al., 2014; Meierjohann, 2014). Therefore, ROS have been generally considered to directly toxic to cell. However, recent studies have demonstrated that ROS play a role as second messengers to regulate mitogen-activated protein kinase (MAPK) in various cells (Son et al., 2013; Tormos et al., 2013), MAPK family constitutes important mediators of signal transduction processes. The ERK1/2 pathway is regulated mostly by mitogenic stimuli, and leads to the production of proteins required for cell growth and differentiation (Munshi and Ramesh, 2013; Liu et al., 2014). In contrast, JNK and p38 are activated primarily by various stresses and are involved in cell transformation, stress responses and apoptosis (Haberzettl and Hill, 2013; Lim et al., 2013; Darling and Cook, 2014; Tian et al., 2014b). Recently, some experiments have shown that paraquat-induced ROS production participates in the phosphorylation of p38, ERK or JNK MAPK (Miller et al., 2007; Ding et al., 2009; Wang et al., 2014c).

Although paraquat has been implicated as a ROS inducer, its mechanisms are largely unknown. In the present study, we investigated the mechanism of paraquat-induced apoptosis in NIH3T3 cells. The results demonstrate that although ERK, JNK, and p38 were all found to be activated in response to paraquat treatment, only ERK activity is important in mediating paraquat-induced apoptosis through a cytochrome C re-
lease-dependent mechanism.

**MATERIAL AND METHODS**

**Reagents and antibodies**
Paraquat (PQ), N-acetylcystein (NAC) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). The MEK inhibitor (U0126), the p38 inhibitors (SB203580) and all were obtained from CalBiochem (San Diego, CA, USA). The anti-Cytochrome C antibody was purchased from Transduction Laboratories (Lexington, KY, USA). The Elk and ERK1/2 antibodies were from Cell signaling Technology (Beverly, MA, USA).

**Cell cultures**
The NIH3T3 mouse embryo fibroblast cell line was obtained from the ATCC (Manassas, VA, USA) and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml (Invitrogen, Carlsbad, CA, USA). They were cultured at 37°C in a humidified chamber containing 5% CO₂.

**Plasmid constructs and oligonucleotides**
- pRL-Luc plasmid was purchased from Promega (San Diego, CA, USA).
- Gal4-c-Jun, Gal4-CHOP and Gal4-Elk1 plasmid were purchased from Stratagene (La Jolla, CA, USA).
- JNK antisense (AS) oligonucleotides used in this study were synthesized at ISIS Pharmaceuticals, Inc. (Carlsbad, CA, USA). The sequences of the oligonucleotides used are as follows: Control (ISIS 15354), TCAGTAATGCCCACATGG; JNK1 AS (ISIS 15347), CTTGTAGGCCCCCTTGG; JNK2 AS (ISIS 15354), GTCCGGGCCAG-GCCAAAGTC. All oligonucleotides were 2'-O-methoxyethyl chimer containing five 2'-O-methoxyethyl-phosphodiester residues flanking a 2'-deoxynucleotide-phosphorothioate residues (Bost et al., 1997).

**Transfection and luciferase activity assay**
To monitor MAPK activity levels, we used luciferase assay based on the fusion proteins that include GAL4 DNA binding domain fused to the activation domain of specific transcription factors that, in turn, drive the expression of firefly luciferase reporter gene. An expression plasmid encoding fusion protein GAL4-Elk (pFA-Elk for ERK activation), GAL4-CHOP (for p38 activation) or GAL4-c-Jun (for JNK activation) was used. In all transfection, a plasmid encoding renilla luciferase was co-transfected, and firefly luciferase activity was normalized to renilla luciferase activity. Dual luciferase activity in the cell extracts was determined according to manufacturer's instruction (Promega, Madison, WI, USA). Briefly, each assay mixture contained 20 μl cell lysate and 100 μl luciferase measuring buffer (LAR II, Promega) and Firefly luciferase and Renilla luciferase activity was measured by luminometer (Glomax, Promega). The luciferase activity data were normalized to the Renilla value, and the results were represented as the average and standard deviation (S.D.) from triplicate of experiments.

**Pl staining**
Cells were collected 24 h following treatment, fixed in 70% ethanol, and stained with propidium iodide (PI, 50 μg/ml) after RNA digestion. PI-stained 10,000 cells were analyzed for DNA content with a FACSscan flow cytometer (Becton Dickinson, San Jose, CA, USA).

**ERK kinase assay**
The cells were lysed and sonicated in a buffer containing Tris (10 mm, pH 7.5), NaCl (150 mM), EGTA (2 mM), orthovanadate (1 mM), DTT (2 mM) and protease inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml) and phenylmethane-sulfonyl fluoride (PMSF) (1 mM) for 30 min at 4°C. Activity was assessed using p44/p42 MAP kinase assay kit (Cell Signaling Technology, Inc.). Briefly, the lysates were immunoprecipitated with immobilized phospho-p44/p42 MAP kinase monoclonal antibody for 5 h at 4°C and the immune complexes were washed three times with lysis buffer, once with kinase buffer, and resuspended in kinase buffer containing Elk-1 fusion protein. The reactions were incubated for 30 min at 30°C and terminated by the addition of SDS sample buffer and analyzed by immunoblotting with anti-phospho-Elk-1 antibody and ERK1/2 antibody. The antigen antibody complexes were visualized by chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Real time PCR analysis was performed for quantification of ERK using the Mx3000P (Stratagene, La Jolla, CA, USA).

**Release of cytochrome C**
Approximately 5×10⁵ cells were trypsinized and collected by centrifugation and the resultant pellets were washed with PBS and resuspended in 100 μl buffer containing 250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF. The cells were then homogenized with 15 strokes of a Teflon homogenizer on ice, and the resulting homogenates were centrifuged at 1000 g for 10 min at 4°C. The supernatants were further centrifuged at 15000 g for 20 min. The resulting supernatants were reserved as the cytosolic fraction and used for Western blot analysis with anti-cytochrome C monoclonal antibody (BD Biosciences, San Jose, CA, USA).

**Determination of DNA fragmentation**
After treatment, cells were harvested by scraping, washed twice with ice-cold PBS, and lysed in lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, and 0.2% Triton X-100) on ice for 20 min. After centrifugation, the supernatant was incubated with RNase A (200 μg/mL) at 37°C for 1 h, then incubated with proteinase K (1 mg/mL) with 1% SDS solution at 50°C for 2 h. The soluble DNA was extracted with phenol, ethanol precipitation, and resuspended in TE buffer. DNA was loaded on 1.5% agarose gel, which stained with ethidium bromide after migration.

**Statistical analysis**
Data in all experiments are represented as mean ± S.D. Statistical comparisons were carried out using two-tailed paired t-test. We considered p<0.05 (indicated * in figures) as significant. Analyses were carried out with Excel (Microsoft).

**RESULTS**

**Paraquat induces apoptosis in NIH3T3 cells**
Accumulation evidences indicate that paraquat lead to cell death by the induction of apoptosis (Han et al., 2014; Wang et
Although several prior studies have investigated the mechanism of paraquat-induced apoptosis, however, it is not fully understood. In the present study, we therefore attempted to investigate the mechanism of paraquat-induced apoptosis in NIH3T3 cells. After exposure to 0.1, 0.25, 0.5, or 1 mM paraquat, sub-G1 DNA content and DNA fragmentation were evaluated in NIH3T3 cells. Fig. 1A showed that paraquat caused apoptosis of NIH3T3 cells in a dose-dependent manner, with a concentration of 1 mM paraquat resulting in death of greater than 80% of the cell population by 24 h of treatment. The intensity of paraquat-induced DNA ladders increased with increasing dose of paraquat in NIH3T3 cells (Fig. 1B). These data indicate that paraquat is able to induce apoptosis in a dose-dependent manner in NIH3T3 cells.

**ERK signaling pathway contributes to paraquat-induced apoptosis**

It is well established that MAPK signaling pathway mediates stress induced apoptosis (Kumar et al., 2014; Wang et al., 2014b). Therefore, we further analyzed the relation of MAPK signal in paraquat-induced apoptosis. We first investigated whether paraquat treatment led to ERK activation. NIH3T3 cells were cotransfected with Gal4-Elk-firefly luciferase vector and control renilla luciferase vector (pRL-Luc). Different dose of paraquat were then treated for 12 h. The cells were lysed, and luciferase activity was measured. Firefly luciferase reading was normalized to that or the control renilla luciferase. Results are shown as means ± S.D. (n=3). *p<0.05. (B) Upper panel, cells were treated with the different dose of paraquat for 12 h. Lower panel, cells were treated with 1 mM paraquat for indicated time. Activation of ERK1/2 was determined by an immune complex kinase assay using Elk-1 fusion protein as substrate. ERK1/2-induced phosphorylation of Elk-1 was measures by immunoblotting with phosphor-Elk-1(Ser383) antibody. The ERK1/2 protein levels were shown using immunoblotting of ERK1/2 as control of immunocomplex.
levels were monitored using antibody capable of recognizing unphosphorylated forms of the proteins. As shown in Fig. 2B, 
1 mM paraquat, which resulted in significant apoptosis, led to 
strong activation of ERK. Activation was apparent at about 30 
min following treatment with 1 mM paraquat and persisted for 
at least 24 h.

To evaluate the functional consequence of ERK activation in 
paraquat-induced apoptosis, we used commercially available 
MEK1/2 inhibitory compound U0126, which are highly sele-
tive in its inhibition of ERK pathway. We observed that pre-
treatment of NIH3T3 cells with 20 µM U0126 totally abolished 
ERK phosphorylation in response to paraquat treatment (Fig. 
3, right panel). Paraquat-induced apoptosis was significantly 
reduced when cells were pretreated with U0126 for 30 min 
before addition of 1 mM paraquat, and this protective effect 
of the MEK inhibitors was dose-dependent and occurred with 
doses expected to suppress ERK activation (Fig. 3, left panel).

**JNK and p38 MAPK signaling pathway are not related 
with paraquat-induced apoptosis**

JNK (c-Jun N-terminal kinase) and p38 have been impli-
cated in stress-related responses and the induction of apopto-
sis. Therefore, we investigated whether JNK and p38 involved 
in the induction of paraquat-induced apoptosis. To compare 
the patterns of activation of the JNK and p38 pathways in re-
spose to paraquat in NIH3T3 cells, cells were cotransfected 
with either Gal4-c-Jun-firefly luciferase vector for measuring 
JNK activation, or Gal4-CHOP-firefly luciferase vector for measuring p38 kinase activation and pRL-Luc following ex-
posure to different dose of paraquat for 12 h, and then cells 
were harvested and luciferase activities were measured. The 
results, shown in Fig. 4A, B, demonstrated that both p38 and 
JNK were activated in response to paraquat treatment. To 
investigate the functional consequences of p38 and JNK ac-
tion, paraquat-induced apoptosis after prevention of JNK 
and p38 was measured. NIH3T3 cells were pretreated with 
p38 specific inhibitor, SB203580 or transiently transfected 
with 0.2 µM each antisense JNK1 and JNK2 oligonucleotides 
(JNK1+JNK2AS), which was phosphorothioate oligonucle-
otides targeted to JNK1 and JNK2 mRNA to block JNK/SPAK 
pathway. As shown in Fig. 4C, the JNK1+JNK2AS-transfect-
ing NIH3T3 cells and the treatment of cells with SB203580 
during exposure to paraquat did not prevent paraquat-induced 
apoptosis. These results indicate that although JNK and p38 
were activated in response to paraquat, neither JNK nor p38 
plays a role in regulating paraquat-induced apoptosis of NIH 
cells. Taken together, among of the three MAPKs, only ERK 
appears to play a major role in influencing the survival of para-
quat-treated NIH3T3 cells.

**ERK MAPK is mediated with release of cytochrome C in 
paraquat-treated cells**

Apoptosis have been described in two major pathways. One 
pathway is extrinsic apoptosis pathway by external re-
ceptor-dependent stimuli. The ligands, such as FASL, TRAIL 
or TNF, interact with their receptors, and then adaptor mol-
ecule, FADD (Fas-associated death domain protein), is as-
associated with death receptors, and subsequently activates 
caspase-8 leading to activation of downstream effector cas-
pases, thus inducing apoptosis (Lavrik and Krammer, 2012; 
Nikoletopoulou et al., 2013). The second pathway is intrinsic 
apoptosis pathway that is mitochondria-dependent and results

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Nikoletopoulou, E., et al. (2013). *Biomol Ther.* 21, 503-509.
The activation of ERK1/2
Intracellular ROS production by paraquat requires the paraquat-induced apoptosis. The MEK inhibitors to diminish this effect suggests that the ERK signaling pathway functions upstream of cytochrome C release in the paraquat-induced apoptosis. The ability of the MEK inhibitors to diminish the extent of paraquat-induced apoptosis was measured 24 h later. Results are shown as means ± S.D. (n=3). *<0.05.

We next investigated whether the intracellular ROS production induced by paraquat could stimulate ERK activation. The NIH3T3 cells were pretreated with NAC for 12 h and the medium was replaced with fresh medium in the presence or absence of 1 mM paraquat and the ERK activity was measured. The results, shown in Fig. 6B, demonstrated that the inhibition of intracellular ROS generation using 5 mM NAC led to markedly decrease in paraquat activation of ERK, suggesting that intracellular ROS, which was produced by paraquat, contributes to the ERK activation in the NIH3T3 cells.

**DISCUSSION**

Many studies have reported that paraquat was a potent inducer of intracellular ROS, which have a critical role in paraquat-induced cell death. In addition to ROS modulate many downstream signaling pathway, including NF-κB, p38 MAPK and mitochondrial pathway (Nahirnyj et al., 2013; Han et al., 2013; Landes and Martinou, 2011; Monian et al., 2013; Son et al., 2011; Han et al., 2011; Nahirnyj et al., 2013; Han et al., 2013). Therefore, paraquat-induced apoptosis is required for paraquat-induced apoptosis in NIH3T3 cells.
which will lead to the development of specifically targeted signaling pathway will require additional study. Characterization of paraquat-induced apoptosis in NIH3T3 cells. Identification of ERK activation, which may, at least in part, involve in the raising the intracellular ROS induced by paraquat stimulation of cell survival. Surprisingly, ROS generation and cytochrome C release in paraquat-treated NIH3T3 cells.

The importance of MAPK signaling pathways in regulating apoptosis during conditions of stress has been widely investigated. Many prior studies have provided evidence indicating that the ERK1/2 pathway is regulated mostly by mitogenic stimuli and leads to the production of proteins required for cell growth and differentiation (Craig et al., 2008; Kim and Choi, 2010; Munshi and Ramesh, 2013). However, more recently, several studies have demonstrated that inhibition of ERK signaling leads to increased sensitivity of anticancer drug (Ohnichi et al., 2005; Tian et al., 2014; Tian et al., 2014b). ERK activation induces the development of B cell (Gold, 2008) and activation of ERK is also involved in the induction of apoptosis in neuronal and various cancer cells (Agrawal et al., 2014; Deschenes-Simard et al., 2014; Esmaeili et al., 2014). Such differential effects of ERK pathway could reflect cell type- and extracellular stress-specificity. In the present studies using NIH3T3 cells, we have provided evidence that activation of ERK is important for the induction of paraquat-induced apoptosis in NIH3T3 cells. Paraquat treatment resulted in high and sustained activation of ERK in these cells. We also found that down-regulation of ERK led to an inhibition of paraquat-induced apoptosis.

In summary, paraquat treatment led to apoptosis, which was associated with release of cytochrome C from the mitochondria, and increase ERK1/2 activity. In addition, the MEK specific inhibitor, U0126, was quite effective in protecting NIH3T3 cells against paraquat-mediated apoptosis. The correlation of removing intracellular ROS with increased cell survival as well as decreased ERK activity after exposure to paraquat suggest that raising the intracellular ROS induced by paraquat stimulates ERK activation, which may, at least in part, involve in the paraquat-induced apoptosis in NIH3T3 cells. Identification of the downstream target of the ROS-cascade in the paraquat-signaling pathway will require additional study. Characterization of this pathway will contribute to the understanding about important signaling pathway of paraquat-induced apoptosis, which will lead to the development of specifically targeted drugs to achieve attenuated paraquat toxicity.

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CONFLICT OF INTEREST

None declared.

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