2007년 2월

석사학위논문

Paraquat induces apoptosis of NIH3T3 cells: Involvement of ERK activation

조선대학교 대학원

의학과

김 낙 천

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파라쿼트에 의한 세포 사멸사 유발 기전연구

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지도교수 유 호 진

이 논문을 의학석사 학위신청논문으로 제출함.

2006 년 10 월 일

조선대학교 대학원

의학과

김 낙 천

김 낙 천의 석사학위논문을 인준함

위원장　조선 대학교　교수 장 인 엽　인

위　원　조선 대학교　교수 전 제 열　인

위　원　조선 대학교　교수 유 호 진　인

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조선대학교 대학원

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파라쿼트에 의한 세포 사멸사 기전연구

김 낙 천

(지도교수 : 유 호 진)

조선대학교 대학원 의학과

파라쿼트는 활성 산소를 생성 시켜 세포 사멸에 관여한다고 알려져 있다. 하지만 파라쿼트에 의한 세포 사멸사 기전에 대해서는 아직 많이 알려진 봐가 없다. 따라서 본 연구에서는 파라쿼트에 의한 세포 사멸사의 기작을 연구하기 위하여 세포 신호 전달에 관여하는 MAPK 의 활성 변화가 파라쿼트에 의한 세포 사멸사에 관여하는지를 관찰하였다. 그 결과 본 연구에서 사용한 NIH3T3 세포에서 파라쿼트투여후 extracellular signal-regulated protein kinase (ERK)의 인산화가 변한다는 사실을 관찰 하였다. 또한 ERK 억제제인 U0126 을 전 처치 하였을 경우에는 파라쿼트에 의한 세포 사멸사 및 세포사멸사를 유발하는 cytochrome C 가 mitochondria 에서 세포질로 분비되는 것을 억제 하였다. 이상의 연구결과 파라쿼트는 세포내 ERK 활성을 증가 시켜 세포 사멸사에 관여할 것으로 사료된다.

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 I demonstrate that extracellular signal-regulated protein kinase (ERK) is required for paraquat -induced apoptosis of NIH3T3 cells. Paraquat treatment resulted in activation of ERK, and U0126, inhibitors of the MEK/ERK signaling pathway, prevented apoptosis. 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.

INTRODUCTION

Paraquat, a redox-cycling compound, known to be pneumotoxin (1). Paraquat is also able to induce behavioral and neurological disorders such as Parkinsonism (2-4). The molecular mechanisms responsible for the cytotoxicity effects of paraquat in cells are not fully understood but are mainly attribute to their ability to generation of reactive oxygen species (ROS) and subsequent interactions with intracellular marcromolecules such as lipids, proteins, and nucleic acids to trigger apoptosis (5,6). 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 disease such as cancer, neurodegeration and ageing (7,8). 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 (9-11). MAPK family constitute important mediators of signal transduction processes that serve to coordinate the cellular response to a variety of extracellular stimuli. Three major mammalian MAPK subfamilies have been described: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 kinases. The ERK1/2 pathway is regulated mostly by mitogenic stimuli, and

leads to the production of proteins required for cell growth and differentiation (12-14). In contrast, JNK and p38 are activated primarily by various stress and are involved in cell transformation, stress responses and apoptosis (15-17).

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 release-dependent mechanism.

MATERIAL AND METHODS

Materials. Paraquat, propidium iodide were purchased from Sigma. The MEK inhibitor (U0126), the p38 inhibitors (SB203580) and were all obtained from CalBiochem (San Diego, CA). The anti-cytochrome c antibody were purchased from Transduction Laboratories (Lexington, KY).

Cell cultures. The NIH3T3 mouse embryo fibroblast line was obtained from the ATCC (Manassas, VA) and were 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 (Life Technologies, Inc.). They were cultured at 37°C in a humidified chamber containing 5% CO_2.

Plasmid constructs and oligonucleotides. pRL-Luc plasmid was purchased from Promega (San Diego, CA). Gal4-cJun, Gal4-CHOP and Gal4-ElK1 plasmid were purchased from Stratagene (La Jolla, CA). JNK antisense (AS) oligonucleotides used in this study were synthesized at ISIS Pharmaceuticals, Inc. (Carlsbad, Calif.). The sequences of the oligonucleotides used are as follows: Control (ISIS 17552), TCAGTAATAGCCCCACATGG; JNK1 AS (ISIS 15347), CTCTGTAGGCCCGCTTGG; JNK2 AS (ISIS 15354), GTCCGGGCCAG-GCCAAAGTC. All oligonucleotides were 2'-O-methoxyethyl chimers containing five

2'-O-methoxyethyl-phosphodiester residues flanking a 2'-deoxynucleotide-phosphorothioate region (18).

Transfection and luciferase activity assay. Dual luciferase activity in the cell extracts was determined according to manufacture's instruction (Promega). Briefly, each assay mixture contained 20 μl cell lysate and 100 μl firefly luciferase measuring buffer (LAR II^R, Promega) and firefly luciferase and renilla luciferase activity was measured by luminometer. Renilla luciferase activity was used to normalize transfection efficiency. All transfections were performed in duplicate, and all were repeated at least three times.

PI 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 FACScan flow cytometer (Becton Dickinson, San Jose, CA)

ERK kinase assay. The cells were lysed and sonicated in a buffer containing Tris (10mm, pH 7.5), NaCl (150 mM), EGTA (2 mM), orthovanadate (1 mM), DTT (2 mM) and protease inhibitors: aprotinine (10 μg/ml), leupeptin (10 μg/ml) and Phenylemethanesulfonyl fluoride (PMSF) (1 mM) for 30 min at 4 ℃. Activity was assessed using p44/42 MAP kinase assay kit (Cell Signaling Technology, Inc.). Briefly,

the lysates were immunoprecipitated with immobilized phopho-p44/42 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-phopho-Elk-1 antibody. The antigen antibody complexes were visualized by chemiluminescence (Amersham Pharmacia Biotech).

Release of cytochrome c. Approximately 5 x 10 6 cells were trypsinized and collected by centrifugation and the resultant pellets were washed with PBS and resuspended in 100 μl buffer containing250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, 1mM dithiothreitol, 1mM PMSF. The cells were then homoginized with 15 strokes of a Teflon homogenizer on ice, and the resulting homogenates were centrifuged at 1000g for 10 min at 4 ℃. The supernatants were further centrifuged at 15000g for 20 min. The resulting supernatants were reserved as the cytosolic fraction and used for Western blot analysis with anti-cytochrome c monoclonal Ab (PharMingen) antibodies.

Determination of DNA fragmentation. After treatment, cells were harvested by scraping, washed twice with ice-cold PBS, and lysed in lysis buffer (10mM Tris-HCl

pH 8.0, 10mM 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 ℃ for 1 h, then incubated with proteinase K (1 mg/mL) with 1% SDS solution at 50 ℃ 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.

Measurement of ROS The ROS were measured using previously described method (27) with some modification. Briefly, Cells were plated at 1×10^5/plate in 60-mm dishes and treated for the indicated times. Cells were harvested with trypsin/EDTA, washed once in PBS, and resuspended in 5 μg of 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) /ml in Hanks' balanced salt solution. Samples were incubated for 10 min at 37 °C and The DCF fluorescence intensity was measured by a fluorescence plate reader (Bio-Tek, FL600) (excitation wavelength, 485 nm; emission wavelength, 530 nm).

RESULTS AND DISCUSSION

Induction of apoptosis by paraquat in NIH3T3 cells. Accumulation evidences indicate that paraquat lead to cell death by the induction of apoptosis (2-6). 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 using NIH3T3 cells. After exposure to 0.1, 0.25, 0.5, or 1 mM paraquat, sub-G_1 DNA content and chormatin 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. To define signaling pathway that mediate paraquat-induced apoptosis in NIH3T3 cells, we evaluated the contribution of the ERK. We first investigated whether paraquat treatment led to ERK activation. NIH3T3 cells were cotransfected with Gal4-ElK1, which contains the Gal4 DNA binding domain fused to the Elk-1 carboxyl-terminal

transactivation domain, and pRL-Luc, which contains the renilla luciferase gene, and fresh medium containing different dose of paraquat was then added to cells. The cells were harvested 12 h later, and the luciferase activities were determined by a luminometer. ElK1 is a transcriptional factor that is activated in response to activation of mitogen activated protein kinase (MAPK) and the renilla luciferase plasmid (pRL-Luc) was used to normalize the transfection efficiency. As shown in Fig. 2A, luciferase activity was elevated by 0.1 mM paraquat and increased in a dose-dependent manner. To confirm the phosphorylation of ERK by paraquat, NIH3T3 cells were exposed to different dose of paraquat for various lengths of time, and ERK activation was then assessed by measuring its kinase activity using an immunocomplex kinase assay with Elk-1 fusion protein as a substrate, and by examining its degree of phosphorylation by Western blot analysis with anti-phospho Elk-1 antibody. Total ERK protein levels was 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 1mM paraquat and persisted for at least 24 h.

To evaluate the functional consequence of ERK activation in paraquat-induced NIH3T3 cell apoptosis, we used commercially available MEK1/2 inhibitory compound

U0126, which are highly selective in its inhibition of ERK pathway. We observed that treatment of NIH3T3 cells with 20 μM U0126 totally abolished ERK phosphorylation in response to paraquat treatment (Fig.3, lower panel). Paraquat-induced apoptosis was significantly reduced when cells were pretreated with U0126 for 30 min prior to addition of 1mM paraquat, and this protective effect of the MEK inhibitors was dose-dependent and occurred with doses expected to suppress ERK activation (Fig. 3, upper panel). 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 (15-17). However, more recently, several studies have demonstrated that inhibition of ERK signaling leads to increased sensitivity of anticancer drug (19,20) and ERK activation is involved in the development of apoptosis in B lymphoma cells (21), neuronal cells (22), HeLa and human lung A549 cells (23). 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.

JNK(cJun N-terminal kinase) and p38 have been implicated in stress-related responses and the induction of apoptosis. Therefore, we investigated whether JNK and p38 involved in the induction of paraquat-induce apoptosis. To compare the patterns of activation of the JNK and p38 pathways in response to paraquat in NIH3T3 cells, cells were cotransfected with either Gal4-cJun for measuring JNK activation, or Gal4-CHOP for measuring p38 kinase activation and pRL-Luc following exposure to different dose of paraquat for 12 h, and then cells were harvested and luciferase activities were measured. The results, shown in Fig. 4A and B, demonstrated that both JNK and p38 were activated in response to paraquat treatment. To investigate the functional consequences of JNK and p38 activation, 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 oligonucleotides targeted to JNK1 and JNK2 mRNA to block JNK/SPAK pathway. As shown in Fig. 4C, the JNK1+JNK2AS-transfecting 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, of the three MAPKs, only ERK appears to play a major role in influencing the survival of paraquat -treated NIH3T3 cells.

Role of ERK in mediating cytochrome c release in paraquat-treated cells. Two major pathways of apoptosis have been described in recent years. One pathway is Fas/APO-1-dependnet. Fas/APO-1 interacts with the receptor-associated death protease caspase-8 leading to activation of downstream effector caspases (24, 25). The second pathway of apoptosis is mitochondria-dependent and results from release of cytochrome c leading to caspase-9 activation through the apoptotic protease-activating factor-1 (Apaf-1) (26-29). Therefore, we sought to investigate whether cytochrome c release occurred in response to paraquat treatment, and if so, to determine whether it was dependent on ERK activation. We isolated cytosolic fractions from lysates of NIH3T3 cells treated with paraquat for 24 h in the presence or absence of U0126 (20 μM). Western blot analysis revealed accumulation of cytosolic cytochrome c release in paraquat-induced apoptosis (Fig. 5). Importantly, this process was markedly inhibited in the presence of the MEK inhibitors, U0126, suggesting that ERK activation is required for paraquat-induced cytochrome c release (Fig. 5). In the present studies, we did not observe any change in either Fas or FasL expression in paraquat-treated NIH3T3 cells

(data not shown). We did, however, observe increased levels of cytochrome c in the cytoplasm of paraquat-treated cells relative to untreated cells. These results suggest that cytochrome c release play a role in mediating paraquat-induced apoptosis. The ability of the MEK inhibitors to diminish this effect suggests that the ERK signaling pathway functions upstream of cytochrome c release in the paraquat-induced apoptosis.

Intracellular ROS production by paraquat requires activation of ERK1/ 2. Recent evidence has suggested that ROS stimulate MAPK activities including ERK, p38 and JNK, which are key events in many cellular processes (9-11). Therefore, intracellular ROS production by paraquat treatment was examined to determine whether or not it led to ERK activation, which could be involved in the paraquat-induced apoptosis. To test this possibility, the level of intracellular ROS production in response to paraquat was investigated using DCFHDA. We found that paraquat treatment led to significantly increase the intracellular ROS production, which could be completely blocked by treatment with 5 mM N-acetylcysteine (NAC) (data not shown). To further investigate the enhancement of ROS production by paraquat is involved in the induction of apoptosis, NIH3T3 cells were pretreated with NAC for 12 h. Subsequently, the cells were incubated with 1mM of paraquat for an additional 24 h, and stained with propidium iodide, and the level of apoptosis was measured by FACsan flow cytometry.

The data presented in Fig. 6A shows that NAC was able to significantly prevent paraquat-induced apoptosis, suggesting that intracellular ROS production is required for paraquat-induced apoptosis in NIH3T3 cells.

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.

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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Fig. 1A

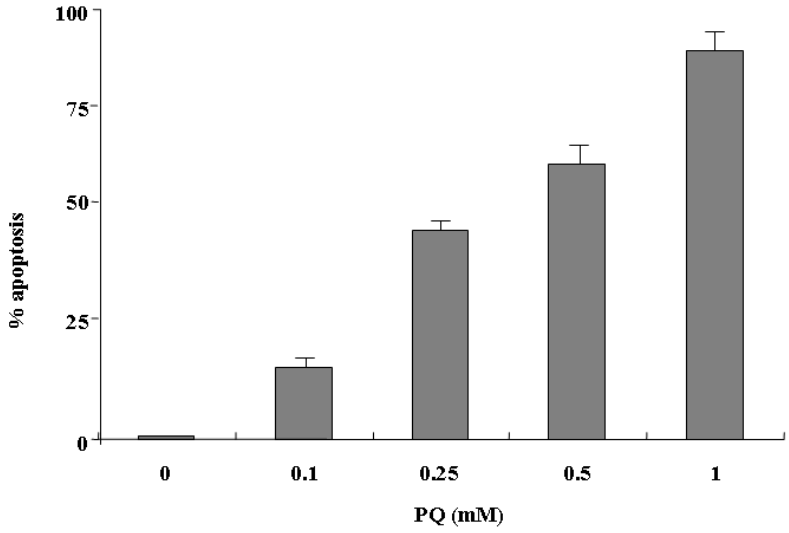


Fig. 1B

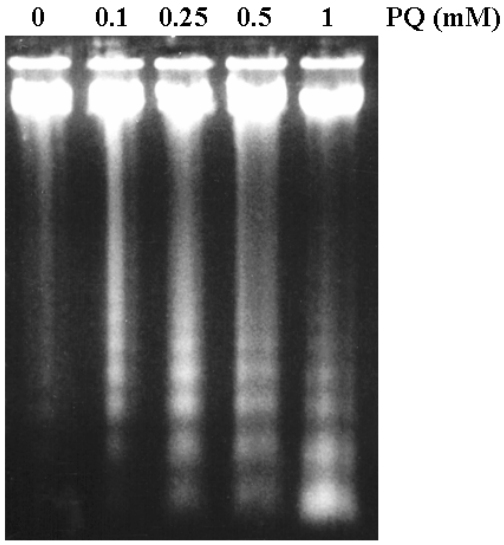


Fig. 2A

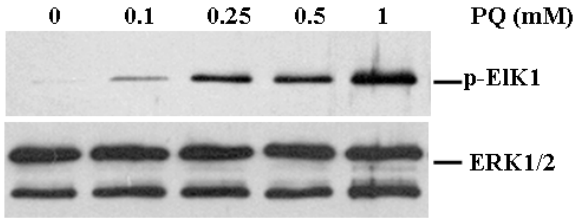


Fig. 2B

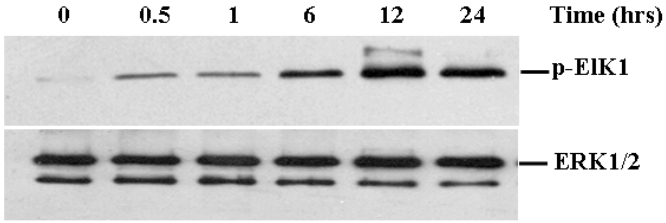


Fig. 3A

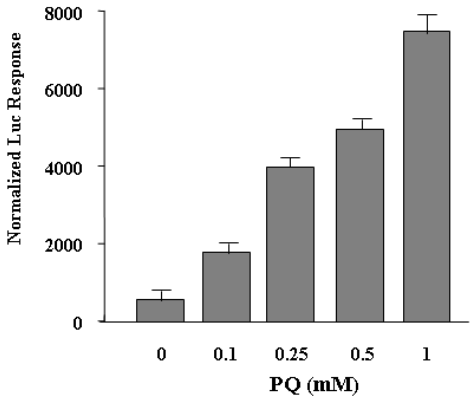


Fig. 3B

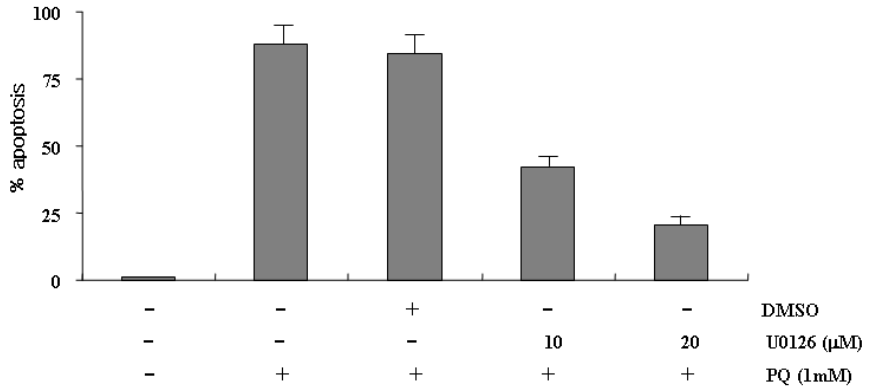


Fig. 3C

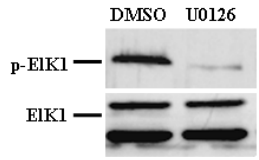
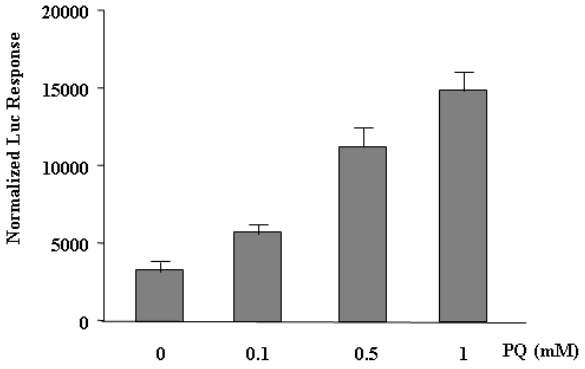


Fig. 4A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10000
5000
0
0
0.1
0.5
1
PQ (mM)

Fig. 4B

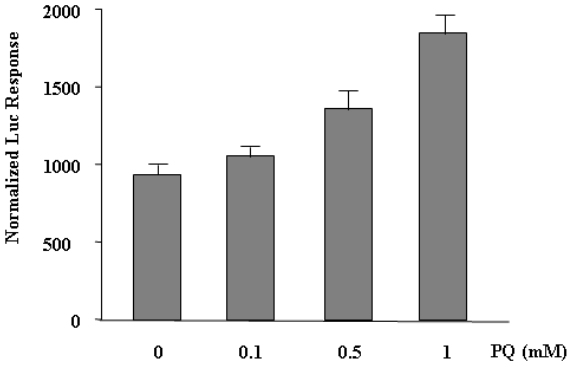
Normalized Luc Response
2000
1500
1000
500
0
0
0.1
0.5
1
PQ (mM)

Fig. 4C

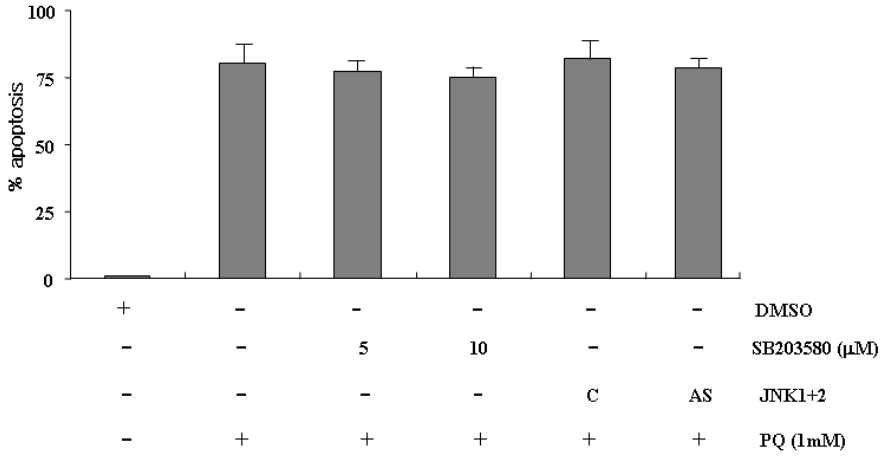
100
75
50
25
0
% apoptosis
+ - - - - - DMSO
- - 5 10 - - SB203580 (μM)
- - - - C AS JNK1+2
- + + + + + PQ (1mM)

Fig. 5

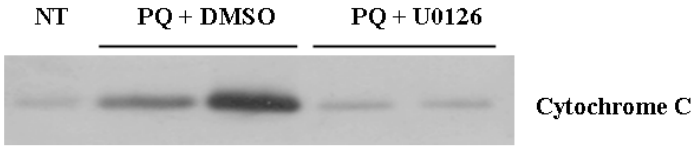
NT
PQ + DMSO
PQ + U0126
Cytochrome C

Fig. 6A

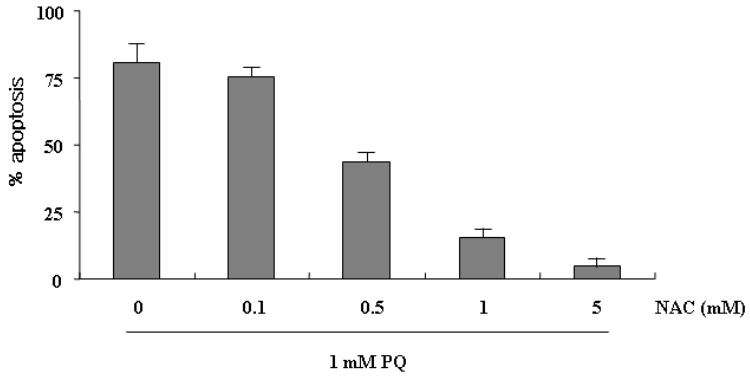
100
75
50
25
0
% apoptosis
0
0.1
0.5
1
5
NAC (mM)
1 mM PQ

Fig. 6B

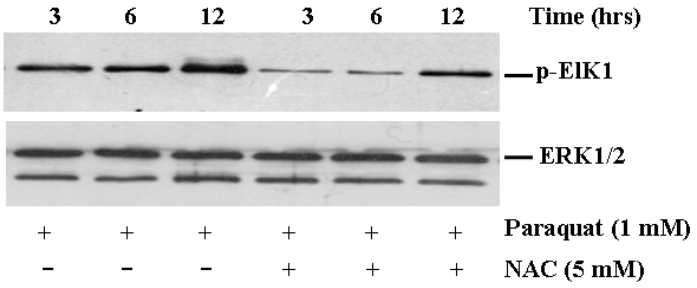
3
6
12
3
6
12
Time (hrs)
p-ElK1
ERK1/2
+ + + + + +
Paraquat (1 mM)
- - - + + +
NAC (5 mM)

FIGURE LEGENDS

Fig 1. Induction of apoptosis by paraquat in NIH3T3 cells. A. Cells were treated with paraquat at final concentration indicated for 24 h. Cells were then stained with propidium iodide and apoptosis examined by flow cytometry. B. Cells were treated with different dose of paraquat for 24 h and DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis. DNA bands were visualized by staining with ethidium bromide.

Fig 2. The effect of paraquat on activation of MEK1/2 MAPKs in NIH3T3 cells. A. Cells were cotransfected with GAL4-Elk and pRL-Luc. Different dose of paraquat were then treated for 12 h. Transfection with pRL-Luc plasmid was used to normalized the transfection. B. Upper panel, cells were treated with the different dose of paraquat for indicated time. Lower panel, cells were treated with 1 mM paraquat for indicated time. Phosphorylation of ERK was determined by an immune complex kinase assay using Elk-1 fusion protein as substrate. Immunostaining of the same blot after striping and reprobing with anti-ERK1/2 antibody is shown.

Fig 3. Inhibition of induction of apoptosis in NIH3T3 cells exposed to paraquat after

pretreatment with the MEK inhibitor, U0126. (A-C) Upper panel, percentage of apoptotic cells in sub-G1 phase. Lower panel, inhibition of ERK1/2 activation by 20 μM U0126 was determined as described in Fig 2B.

Fig. 4. The effect of paraquat on activation of JNK and p38 MAPKs in NIH3T3 cells. Cells were cotransfected with either Gal4-CHOP plasmid (A) or GAL4-cJUN plasmid (B) and pRL-Luc and then cells were treated with different dose of paraquat for 12 h. Renilla luciferase was used to normalized the transfection. C. Cells were preincubated with p38 inhibitors SB203580, or transiently transfected with a combination of JNK1 and JNK2 antisense oligonucleotides (AS) or control oligonuleotide (c), and subsequently treated with I mM paraquat for 24 h, and then the extent of apoptosis was measured.

Fig. 5. MEK inhibitor suppresses cytochrome c release. Cells were pretreated with the U0126 (20 μM) for 30 min and then treated with 1 mM paraquat for 24 h. The cytosolic fraction was separated from mitochondria-enriched fraction and subjected to Western blot analysis with a monoclonal antibody to cytochrome c.

Fig. 6. The effect of ROS on the activation of ERK1/2 induced by paraquat in NIH3T3 cells A. Cells were pretreated with different dose of NAC for 12 h and then treated with 1 mM paraquat. The extent of apoptosis was measured 24 h later. C. Cells were treated with 1mM paraquat for indicated time in the presence or absence of 5 mM NAC, and Phosphorylation of ERK was determined as described in Fig 1B.

저작물 이용 허락서

학 과	의학과	학 번	20047641	과 정	석사
성 명	한글 : 김낙천 한문 : 金洛天 영문 : Kim Nak Cheon				
주 소	전북 전주시 덕진구 덕진동 1가 1434-1				
연락처	E-MAIL : 74@hanmail.net				
논문제목	한글 : 파라쿼트에 의한 세포 사멸사 유발 기전연구 영문 : Paraquat induces apoptosis of NIH3T3 cells: Involvement of ERK activation				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(o) 조건부 동의( ) 반대( )

2006년 12 월 일

저작자: 김 낙 천 (서명 또는 인)

조선대학교 총장 귀하