行政院國家科學委員會專題研究計畫　成果報告

致癌黴菌毒素對於細胞基因表現及訊號傳遞的影響

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Abstract

Patulin (PAT), a mycotoxin produced by certain species of *Penicillium* and *Aspergillus*, is often detectable in moldy fruits and their derivative products. PAT led to. a concentration-dependent and time-dependent increase in phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in human embryonic kidney (HEK293) cells, human peripheral blood mononuclear cells (PBMCs), and Madin-Darby canine kidney (MDCK) cells. Treatment of HEK293 cells with 5 μM and 0.05 μM PAT induced ERK1/2 phosphorylation after 30 min or 24 h, respectively. Treatment of human PBMCs for 30 min with 30 μM PAT also dramatically increased the phosphorylated ERK1/2 levels. The MEK1/2 inhibitor, U0126, but not MEK1 inhibitor, PD98059, suppressed ERK1/2 activation in both HEK293 and MDCK cells. In HEK293 cells, U0126-mediated inhibition of PAT-induced ERK1/2 phosphorylation resulted in a significant decrease in levels of DNA damage, expressed as tail moment values, in the single cell gel electrophoresis assay. Conversely, U0126 did not affect cell viability, lactate dehydrogenase release, and the DNA synthesis rate in PAT-treated cultures. Exposure of HEK293 cells for 90 min to 15 μM PAT elevated the levels of early growth response gene-1 (*egr-1*) mRNA, but not of *c-fos*, *fosB*, and *junB* mRNAs. These results indicate that in human cells, PAT causes rapid and persistent activation of ERK1/2 through the MEK2 pathway and this signaling pathway plays an important role in mediating PAT-induced DNA damage and *egr-1* gene expression.

中文摘要

棒麴毒素(Patulin 簡稱 PAT)是由 *Penicillium* 和 *Aspergillus* 菌屬所分泌的黴菌毒素(mycotoxin)，通常在發霉的水果和再製品中被偵測出來。以棒麴毒素處理人類胚胎腎臟細胞株(human embryonic kidney cell 簡稱 HEK293)、周邊血液單核球細胞(human blood mononuclear cells 簡稱 PBMCs)和 Madin-Darby 氏犬科腎小管細胞株(Madin-Darby canine kidney cell 簡稱 MDCK)時，隨著毒素濃度及處理時間的增加，會使上述細胞中的 extracellular signal-regulated protein kinases 1 and 2(ERK1/2)的磷酸化表現增加。將 HEK293 細胞株分別以 5 μM 及 0.05 μM 的 PAT 處理 30 分鐘或 24 小時，可以誘導 ERK1/2 磷酸化的表達；而在人類 PBMCs 中，以 30 μM PAT 處理 30 分鐘也可以得到 ERK1/2 磷酸化增加的現象。在 HEK293 及 MDCK 細胞珠中使用 MEK1/2 抑制劑-U0126 足以抑制由 PAT 所誘發的 ERK1/2 活化，但是 MEK1 的抑制劑-PD98059 並不具有相同的效果。當利用 HEK293 細胞株進行單細胞電泳實驗時，由 tail moment 所得之數值發現 U0126 藉由抑制 ERK1/2 的磷酸化表現，會顯著的降低 PAT 所誘發之 DNA 損傷的現象。然而 U0126 的處理並不會影響 PAT 所造成的細胞存活率、細胞膜完整性和 DNA 合成速率等現象。以 15 μM 處理 HEK293 細胞株 90 分鐘後，會顯著提高 early growth response gene-1 (egr-1) mRNA 含量，但是並不會改變 *c-fos*，*fos B* 或是 *junB* 的 mRNA 量。以上結果顯示，在人類細胞株中 PAT 會藉由 MEK2 造成 ERK1/2 快速且持續的活化，而這一條訊息傳導的途徑，在 PAT 所誘導 DNA damage 和 *egr-1* 基因的表達中，扮演著重要的角色。

Introduction

Patulin (PAT) (Fig. 1) is a mycotoxin produced by certain fungal species of *Aspergillus* and *Penicillium* which grow on a variety of foods, including fruit and grains. PAT is frequently found as a contaminant of apple juice, apple juice concentrates, and related products and has been reported in other foods, including pears, vegetables, flour, and malt feed. A safety level of 50 μg/l of PAT in apple juice, established by the World Health Organization, is applied in several countries.

Several targets, including the kidney, liver, intestinal tissue, and immune system, are affected by in vivo administration of PAT. In addition to its acute toxic effects, PAT is reported to be carcinogenic, embryotoxic, and teratogenic in certain experimental animals. Some studies have indicated that it is also a mutagen, inducing DNA damage, chromosome aberration, and micronuclei formation in mammalian cells. Recently, we have shown that PAT has a potent ability to cause oxidative damage to DNA in HEK293 cells and human peripheral lymphocytes. It also appears to interfere with the activity of several enzymes and inhibit protein and RNA synthesis in various cellular models. It is generally believed that PAT exerts its cytotoxic and immunosuppressive effects by covalently binding to essential sulfhydryl groups in proteins and amino acids, but little information is available about the specific mechanisms or molecular basis of PAT toxicity in human cells.

Mitogen-activated protein kinases (MAPKs) are important signal-transducing enzymes connecting cell surface receptors to critical regulatory targets within cells. MAPKs have been implicated in a broad spectrum of physiological processes, including cell growth, apoptosis, differentiation, and inflammation. In mammalian cells, there are at least four distinct groups of MAPKs, these being extracellular signal-related kinases (ERK1 and ERK2; respective molecular weights of 44 and 42 kDa), jun amino-terminal kinases (JNK; SAPK1), p38 kinases (SAPK2), and ERK5. The signaling pathways leading to the activation and regulation of different MAPKs are usually biochemically and functionally distinct. In general, the ERK cascade is predominantly activated by mitogenic stimuli, such as growth factors and differentiation signals. In the case of stimulation by epidermal growth factor (EGF) and platelet-derived growth factor, ERK1/2 activation is involved in cell growth, as ERK1/2 phosphorylates and activates numerous substrates involved in gene transcription, nucleotide synthesis, protein synthesis, and cell cycle progression. The ERK pathway is regulated by the sequential activation of the proto-oncogenes, Ras and Raf-1, and the MAPK kinases, MEK1/2, which then activate ERK1/2 by a dual phosphorylation of threonine and tyrosine residues within the catalytic domain of ERK. Phosphorylated ERK1/2 undergoes dimerization and translocates into the nucleus to upregulate the transcriptional expression of certain immediate early genes.

Due to the widespread nature of fungal species, mycotoxins are considered unavoidable contaminants in foods and feed. Some mycotoxins, including trichothecene, ochratoxin A, and fumonisin B_1, have been shown to induce MAPK activation in various cellular models, but no studies have been performed on the intracellular signaling mechanism involved in PAT-induced toxicity. In the present study, we showed that PAT activates the MAPK signaling pathway in a cell line derived from human embryonic kidney cells (HEK293), in human peripheral blood monocytes (PBMCs), and in Madin-Darby canine kidney (MDCK) cells. We also demonstrated that, in HEK293 cultures, inhibition of activated ERK1/2 dramatically reduces PAT genotoxicity; in contrast, phosphorylated ERK1/2 appears not to be involved in cell viability and DNA synthesis.

Materials and Methods

Reagents. Cell culture media and serum were obtained from Life Technologies (Grand Island, NY). Kinase inhibitors (MEK1 inhibitor, PD 98059, and the MEK1/2 inhibitor, U0126) and polyclonal rabbit antibodies against phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were obtained from Pierce (Rockford, IL). PAT and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). PAT was dissolved at a concentration of 10 mM in 15% ethanol and stored at -20°C.

Cell cultures and regents. Both HEK293 and MDCK cells were obtained from the Bioresources Collection and Research Center, Taiwan. HEK293 cells were maintained in minimal Eagle's medium (MEM) supplemented with 10% horse serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin at 37℃ in a humidified 5% CO_2 incubator. MDCK cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics as described above. Human PBMCs were isolated from the heparinized venous blood of five healthy subjects by density-gradient sedimentation over Ficoll-Paque (Amersham Biosciences) and then cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and 1.5% phytohemagglutinin at 37℃ for 48-72 h before toxin treatment.

***Preparation of whole cell extracts*.** Cells (5×10^5 on a 5 cm tissue culture plate) were cultured for 72 h in medium containing 10% of the appropriate serum, and then serum-starved by transfer to 1% serum for 18 h. The serum-starved cells at 80% confluency were then exposed to various concentrations of PAT or vehicle (15% ethanol in PBS) for the designated time. In experiments to determine the effects of protein kinase inhibitors, serum-starved cells were pretreated for 30 min with PD98059 or U0126 before addition of PAT or vehicle in the continued presence of the inhibitor.

PAT-treated or vehicle-treated cells were rinsed with 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.5, (PBS) and lysed by addition of extraction buffer (PBS containing 5% glycerol, 1 mM DTT, 1 mM EDTA, pH 8.0, 0.5% Triton X-100, 0.8 μM aprotinin, 1 mM AEBSF, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstain A, 14 mM E-64, and 1 mM phenylmethylsulfonyl fluoride). The cell lysate was kept on ice for 10 min, and then centrifuged at 16,000 g for 20 min at 4°C. The protein concentration of the supernatant solution was determined using the Bradford protein assay (Bio-Rad, Hercules,CA) with bovine serum albumin as the standard.

***Western blot analysis*.** Equal amounts of proteins (40 μg) from each sample preparation were incubated for 3 min at 95°C in Laemmli buffer, separated on a 10 % SDS-polyacrylamide discontinuous gel, and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with PBS containing 10% skimmed milk for 1 h at room temperature, then incubated for 1 h with rabbit polyclonal antibodies against phospho-ERK1/2 or ERK1/2 (1: 1000 dilution), and followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (1: 5000) for another 1 h. Bound antibody on the membrane was detected using an enhanced chemiluminescence detection system according to the manufacturer's manual (Amersham Pharmacia Biotech, Amersham, UK). To re-probe the membrane with another primary antibody, antibodies were stripped for 30 min at room temperature with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) and the membrane was washed three times with PBS-Tween 20. The intensities of bands on blots were quantitated using the ImageGauge program Ver. 3.46 (Fuji Photo Film, Tokyo).

Measurement of lactate dehydrogenase (LDH) release. LDH released into the medium was assayed using a LDH Cytotoxicity Detection Kit (TAKARA BIO Inc.).

HEK293 cells were seeded at 5 x 10^3 cells/well in quadruplicate in 96-well tissue culture plates and allowed to attach for at least 18 h to obtain monolayer cultures, which were then treated for 30 min with vehicle (15% ethanol in PBS) alone or with various concentrations of PAT (final concentration 15-100 μM). An aliquot (100 μL) of cell-free medium from each well was removed for LDH assay according to the manufacturer's protocol.

BrdU incorporation assay. HEK293 cells were seeded at 1x 10^3 cells/well in quadruplicate in 96-well tissue culture plates and allowed to attach for at least 18 h to obtain monolayer cultures. The culture medium was first replaced with 100 μL of MEM containing 1% horse serum with or without 10 μM U0126 for 30 min, and then co-incubation with various concentrations of PAT (0.1-0.5 μM) or vehicle (15% ethanol) for another 24 h. A chemiluminescence immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis, was performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN).

Single-cell gel electrophoresis (SCGE) assay. Standard SCGE assays were conducted using a protocol reported by Wang et al. (2001) according to the guidelines developed by Tice et al. (2000). Briefly, HEK293 (1×10^5 cells) were treated with 10 μM U0126 for 30 min, and then co-incubated with vehicle alone, 7.5 or 15 μM PAT, or 15 μM H_2O_2 for 1 h. The adherent cells were trypsinized, mixed with 1% low-melting-point agarose at 42°C. The mixtures were immediately transferred to CometSlides (Trevigen Inc., Gaithersburg, MD), which were then immersed for 1 h in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA pH 10, 10 mM Tris, 1% sodium lauryl sarcosinate, 1% Triton X-100, and 1% DMSO). After electrophoresis in an alkaline buffer (300 mM NaOH, 1 mM EDTA, pH 13) at 300 mA for 30 min, the DNA on the slides was stained with SYBR green I.

The image of each cell on the slide was visualized and analyzed on a fluorescence microscope (BX51, Olympus) equipped with the computer software from CometAssay Ⅲ (Perceptive Instruments Ltd, UK), which calculates the tail moment value of each cell from the amount of DNA in the tail and the distance of tail migration (Olive et al., 1990). For each experimental point, four cultures were treated independently, and DNA damage levels in 80 cells were measured from each culture.

Gene expression profiling on microarrays. HEK293 cells were cultured in 10 cm tissue culture plates with medium containing 10% serum. Cells with 80% confluence were exposed to vehicle (15% ethanol in PBS) or 15 μM PAT for 90 min, and then total RNA was extracted and purified using Qiagen RNeasy isolation kits (Qiagen , Valencia, CA) according to the manufacturer's protocol. Labeling of cDNAs, preparation of microarrays, and the hybridization reaction were performed as a custom service by eGenomix Inc (Taipei, Taiwan). Briefly, hybridization reactions were carried out on ABC Human Chip 8K-1 slides containing 7537 human cDNA clones (eGenomix Inc.). Fluorescent signals were detected on a GenePix 4000 B (Axon Instruments, Union, CA) and the data were analyzed using Axon GenePix Pro (version 3.0). The experiments were independently performed twice.

Statistical analysis. Values are presented as the mean ± SEM. Statistical differences between the control and treated groups were determined using Student's *t* test and were considered significant at $p<0.05$.

Results and Discussion

Effect of PAT on ERK1/2 activation in various cell cultures

The ability of PAT to activate ERK1/2 was investigated by exposing HEK293 cultures to various concentrations of PAT in medium containing only 1% serum. The ERK1/2 activation was determined with Western blotting using antibodies specific for phospho-ERK1/2. As shown in Figure 2A, exposure of cells to PAT for 30 min resulted in a dose-dependent increase in ERK1/2 phosphorylation. PAT at the concentrations ranging from 5 to 50 μM enhanced the phosphorylated ERK1/2 levels 5.8- to 33.4-fold compared to the solvent-treated control. After treatment of the HEK293 cells for 24 h with low levels of PAT (0.05-1.50 μM), increased ERK1/2 phosphorylation was seen even at 0.05 μM (Fig. 2B). When HEK293 cells were incubated with 15 μM PAT for various times, signals of phospho-ERK1/2 were detected within 15 min and remained high for at least 2 h (Fig. 2C). On the other hand, after treatment of HEK293 cells with 100 ng/ ml of EGF, ERK1/2 activation peaked within 5 min and began to drop substantially by 90 min.

To investigate the upstream activators involved in the PAT-induced signaling pathway, HEK293 cells were exposed to PAT in the presence of either U0126, an inhibitor of the ERK1/2 upstream kinases, MEK1/2, or PD98059, a more MEK1-specific inhibitor. As shown in Fig. 3, U0126 at the concentration of 10 μM completely blocked PAT-induced ERK1/2 phosphorylation, but 20 or 50 μM PD98059 did not have similar effect. These results suggest that, in HEK293 cells, activation of ERK1/2 by PAT is mediated through MEK2.

Since PAT treatment induced ERK1/2 phosphorylation in HEK293 cell cultures, its effect was also examined in human PBMCs and in MDCK cells. When freshly prepared human PBMCs were treated with various concentrations of PAT for 30 min, dose-dependent ERK1/2 phosphorylation was observed (Fig. 4A). Similarly, in MDCK cells, PAT concentrations equal to or higher than 15 μM resulted in a marked increase in phospho-ERK1/2 levels; this effect was inhibited in the presence of U0126 (Fig. 4B).

Effects of U0126 on cell viability and DNA synthesis rate of PAT-treated cells

To examine whether there was a correlation between ERK1/2 activation and PAT cytotoxicity, HEK293 cells were exposed to PAT in the presence of U0126 and then cell viability and plasma membrane damage were determined using the MTT assay or LDH release assay, respectively. Table 1 shows that when HEK293 cells were exposed for 90 min to different concentrations of PAT, no significant cytotoxicity was seen until the dose reached 30 μM; in addition, the presence of U0126 did not modulate the cytotoxicity of PAT. Table 1 also shows that U0126 had no effect on PAT-induced plasma membrane damage.

The role of ERK1/2 activation in DNA synthesis in PAT-treated cells was also studied using the BrdU incorporation assay. BrdU incorporation of HEK293 cells was significantly reduced to 72 and 64 % of control levels following 24 h treatment with 0.3 or 0.5 μM PAT, respectively (Fig. 5). Co-administration of U0126 with PAT did not elevate or reduce the BrdU levels in PAT-treated cultures. These data suggest that activation of the ERK pathway in PAT-treated HEK293 cells does not directly correlate with the cytotoxicity or DNA synthesis rate in PAT-treated HEK293 cells.

Effect of U0126 on PAT-induced DNA damage

We have previously demonstrated by SCGE assays that treatment of human cells with PAT significantly increases the tail moment values, an indicator of cellular DNA

damage (Liu et al., 2003). To explore the role of ERK1/2 activation in PAT-induced DNA damage, HEK293 cells were left untreated or treated with 10 μM U0126 for 30 min before co-exposure to PAT (7.5 and 15 μM) or H_2O_2 (15 μM; positive control) for 1 h, and then subjected to SCGE assays. When HEK293 cells were treated with 7.5 μM PAT alone, the tail moment value (3.93 ± 0.33) was approximately twice that in the untreated control (1.99 ± 0.3) and this value was reduced to 2.19 ± 0.47 in the presence of U0126 (Fig. 6). A similar effect was observed in 15 μM PAT-treated cultures. In contrast, U0126 did not influence the tail moment values induced by 15 μM H_2O_2. These results suggest that activation of the ERK1/2 pathway is involved in the PAT-induced DNA damage.

Induction of Egr-1 gene expression in PAT-treated HEK293 cells

Activation of the ERK1/2 signaling pathway can modulate the transcriptional expression of various immediate early genes in various cell models (Balmanno and Cook, 1999; Hodge et al., 1998). To identify the downstream target genes activated via the PAT-induced ERK1/2 pathway, RNA preparations from HEK293 cultures treated with 15 μM PAT or vehicle for 90 min were subjected to cDNA microarray analysis. As shown in Figure 7, a significant upregulation of early growth response gene 1 (*egr-1*) mRNA levels was seen in PAT-treated cells, whereas levels of transcripts corresponding to *c-fos, fos B, JunB* or the house-keeping gene *gapd* (glyceraldehyde-3-phosphate dehydrogenase) were not affected compared to solvent-treated cultures.

Figure Legends

Fig. 1. Structure of patulin (PAT)

Fig. 2. Concentration-dependent and time-dependent induction of ERK1/2 phosphorylation by PAT in HEK293 cells. Subconfluent HEK293 cells were rendered quiescent by incubation for 18 h in medium containing 1% serum, and then (A) incubated for 30 min with various PAT concentrations (0-50 μM), (B) incubated for 24 h with low doses (0-1.5 μM) of PAT, or (C) incubated with 15 μM PAT and 100 ng/ml of EGF for up to 120 min and 90 min, respectively. Whole cell extracts were prepared immediately after treatment and ERK1/2 activation was estimated by Western blotting using anti-phospho-ERK1/2 antibodies, which detect phosphorylated Thr202 and Tyr204 on both ERK1 and ERK2. The relative phospho-ERK1/2 levels shown in the lower panel of (A) are the mean ± SEM for the densitometric analyses of four independent experiments normalized by arbitrarily setting the value for vehicle-treated cells as 1.

Fig. 3. Effect of U0126 or PD98059 on PAT-induced ERK1/2 activation in HEK293 cells. HEK293 cells in medium containing 1% serum were pretreated with U0126 (1 or 10 μM) for 30 min or PD98059 (20 or 50 μM) for 1 h, and then co-incubated with these inhibitors and either vehicle (15% ethanol in PBS) or 30 μM PAT for another 30 min. Whole cell extracts were prepared immediately and subjected to Western blotting in which phospho-ERK1/2 and ERK1/2 antibodies were used as probes.

Fig. 4. Activation of ERK1/2 by PAT in human PBMCs (A) and MDCK cells (B). The cells were treated for 30 min with various concentrations of PAT (0-50 μM) and then ERK1/2 activation in whole cell extracts was determined by Western blotting using anti-phospho-ERK1/2 antibodies. In the lower panel of (B), MDCK cells were pretreated for 30 min without or with U0126 (1 or 10 μM) and then co-incubated with the same agent plus vehicle or 30 μM PAT for another 30 min before cell extract

preparation and Western blot analysis.

Fig. 5. Effect of U0126 on DNA synthesis in PAT-treated HEK293 cells. HEK293 cells were left untreated (●)or treated with 10 μM U0126 for 30 min (□) and then co-incubated for another 24 h with the same agents plus vehicle or various concentrations of PAT (0.1, 0.3, or 0.5 μM). DNA synthesis levels were measured using the BrdU incorporation assay and expressed as a percentage of that in control cells exposed to vehicle only. The data are given as the mean ± SEM (n = 4).

Fig. 6. Effect of U0126 on PAT-induced DNA damage in HEK293 cells. HEK293 cells were left untreated or treated with 10 μM U0126 for 30 min and then co-incubated with vehicle (15% ethanol in PBS), PAT (7.5 or 15 μM), or H_2O_2 (15 μM) for 1 h. DNA damage levels, expressed as the tail moment value, were determined using the SCGE assay. The data are expressed as the mean ± SEM (n = 6). *, significant difference (p<0.05) compared to the control group treated with neither PAT nor U0126.

Fig. 7. Induction of *egr-1* gene expression by PAT in HEK293 cells. HEK293 cultures with 80% confluence were treated with vehicle (15% ethanol in PBS) or 15 μM PAT for 90 min, and then total RNA was extracted and subjected to cDNA microarray analysis as described in the Materials and Methods. The data are expressed as the mean ± SEM for two independent experiments. *, significant difference compared to *gapd* mRNA levels (p<0.05).

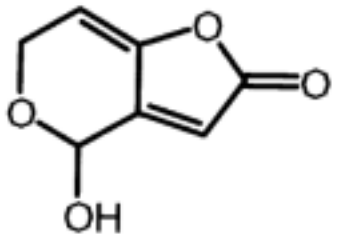
Table 1. Effects of U0126 on PAT-induced cytotoxicity and LDH release in HEK293 cells

PAT (μM)	Cell viability [a] (% of control)		LDH activity [b] (% of control)	
	−U0126	+U0126	−U0126	+U0126
15	101.8 ± 4.8	97.8 ± 4.9	102.3 ± 8.1	104.8 ± 11.4
30	70.4 ± 6.5	77.4 ± 7.8	119.1 ± 10.7	109.8 ± 7.7
50	55.6 ± 2.8	56.8 ± 9.3	127.9 ± 15.2	115.8 ± 7.2
100	38.1 ± 2.2	37.2 ± 5.4	142.7 ± 10.6	145.6 ± 10.6

[a] Cells were exposed to various concentrations of PAT for 90 min. Cell viability was determined by the MTT reduction assay and expressed as a percentage of that of control cells exposed to vehicle only. The data are the mean ± SEM for five independent experiments. No significant difference was found between the cells treated with PAT in the presence or absence of U0126.

[b] Cells were exposed to various concentrations of PAT for 30 min. Plasma membrane damage was determined by measuring LDH activity released in the culture medium and expressed as a percentage of that seen with control cells exposed to vehicle only. The data are the mean ± SEM for five independent experiments. No significant difference was found between the cells treated with PAT in the presence or absence of U0126.

Figure 1



Patulin

Figure 2.

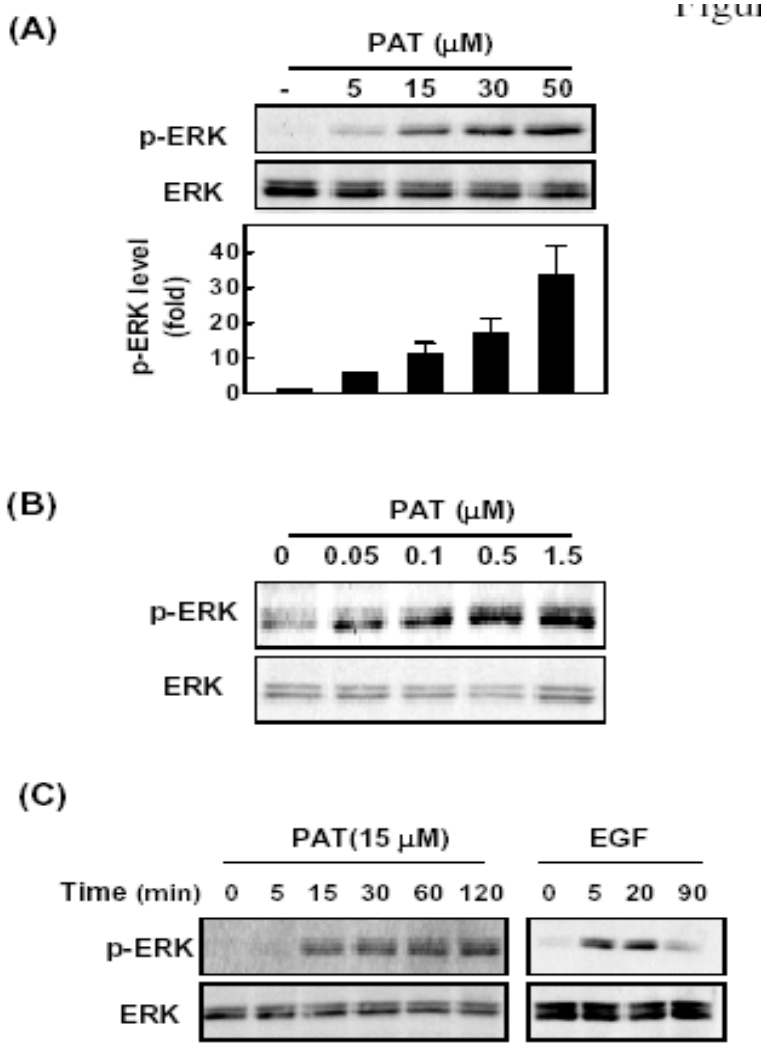


Figure 3

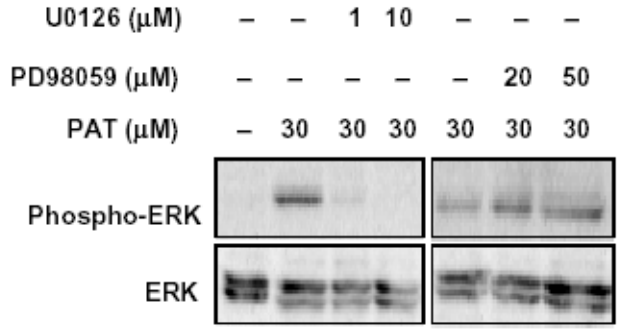


Figure 4

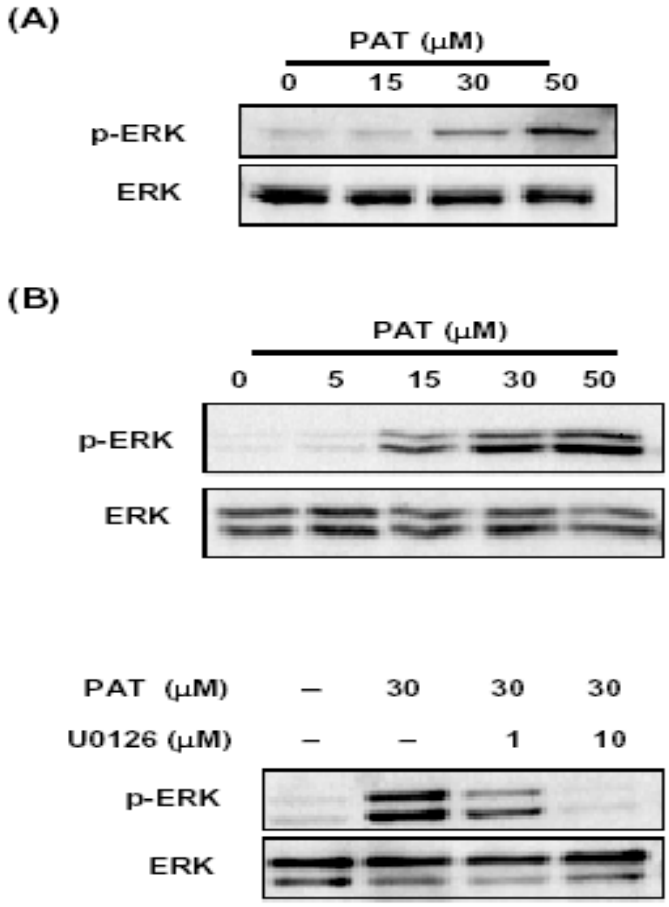


Figure 5

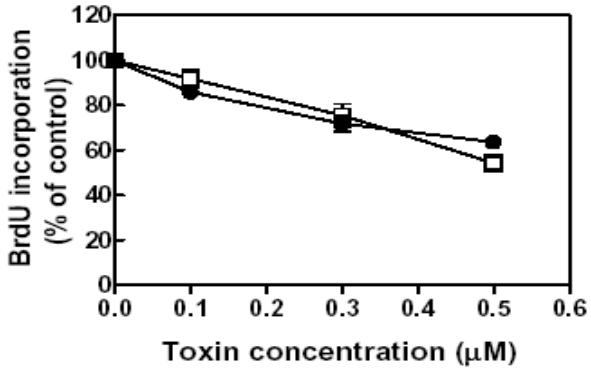


Figure 6

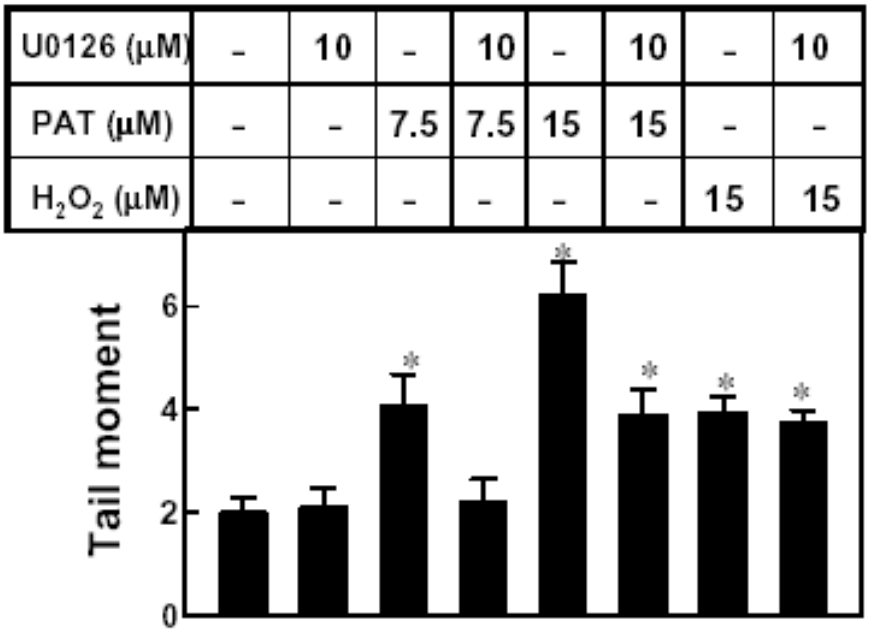


Figure 7