学位論文

Paraquat toxicity is attenuated by 4-phenylbutyrate-induced phosphorylation of ERK2 via PI3K in A549 cells

（4-フェニル酪酸は ERK2 のリン酸化を介してパラコート毒性を抑制する）

保科　千里

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Paraquat toxicity is attenuated by 4-phenylbutyrate-induced phosphorylation of ERK2 via PI3K in A549 cells

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ABSTRACT

Paraquat (PQ) is a widely used herbicide in the world despite being highly toxic to humans. PQ causes fatal damage to multiple organs, especially the lungs. While oxidative stress is the main toxic mechanism of PQ, there is no established standard therapy for PQ poisoning. In this study, we investigated the cytoprotective effect of 4-phenylbutyrate (4PBA) on PQ toxicity in human lung adenocarcinoma A549 cells. Phosphorylation levels of major survival signaling kinases Akt and ERK, as well as expression levels of antioxidant enzymes catalase and superoxide dismutase 2 (SOD2) were examined. The cytoprotective mechanism of 4PBA against PQ was compared with the antioxidant reagent trolox. We demonstrated that both 4PBA and trolox attenuated PQ toxicity, but their mechanisms were different. 4PBA increased ERK2 phosphorylation levels, which could be inhibited by the PI3K inhibitor LY294002. The cytoprotective effect of 4PBA was also inhibited by LY294002. Catalase expression levels were increased by 4PBA, although this increase was not inhibited by LY294002. 4PBA did not increase SOD2 expression. Trolox did not affect phosphorylation of Akt or ERK, or the expression of antioxidant enzymes. These results suggest that 4PBA attenuated PQ cytotoxicity by ERK2 activation via PI3K. Our study may provide new findings for understanding the molecular mechanism underlying cytoprotection by 4PBA, as well as new therapeutic targets for PQ poisoning.

1. Introduction

Paraquat (PQ) is a widely used herbicide in the world despite being highly toxic to humans. Acute toxicity of PQ causes fatal damage to multiple organs, especially the lungs, and causes disorders such as pulmonary fibrosis [1]. While oxidative stress is the main toxic mechanism of PQ [2,3], there is no established standard therapy for PQ poisoning.

We previously reported that sodium tauroursodeoxycholate protects cells from PQ toxicity through chemical chaperone activity [4]. 4-Phenylbutyrate (4PBA) is used for its ammonia-scavenging activity to treat urea cycle disorders [5,6], prevents ER stress by modifying unfolded proteins as a chemical chaperone, and regulates gene expression as a histone deacetylase inhibitor. Notably, these properties are similar to sodium tauroursodeoxycholate [7–9]. While 4PBA has been widely studied as chemical chaperone and histone deacetylase inhibitor in pathological models such as those for Alzheimer's disease, Parkinson's disease and diabetic nephropathy [10–12], its antioxidant action and survival signaling pathway remain controversial; thus, more research is needed [13,14].

In this study, we tried to clarify whether survival signal transduction is involved in the cytoprotective mechanism of 4PBA against PQ toxicity. First, we confirmed the cytoprotective effect of 4PBA against PQ toxicity in the A549 cell line, a commonly used model of alveolar epithelial cells [15–17]. Next, we sought to examine whether 4PBA influenced major cell survival signaling pathways PI3K/Akt and MEK/ERK in the presence of PQ. In addition, expression levels of antioxidant enzymes catalase and superoxide dismutase 2 (SOD2) were examined. Finally, the cytoprotective mechanism of 4PBA against PQ was compared with the antioxidant reagent trolox.

Abbreviations: ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; MEK, MAPK-ERK kinase; MAPK, mitogen-activated protein kinase.

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2. Materials and methods

2.1. Reagents and antibodies

1,1′-Dimethyl-4,4′-bipyridinium chloride (PQ) was purchased from Kanto Kagaku (Tokyo, Japan). Sodium 4-phenylbutyrate (4PBA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trolox, LY294002, and U0126 were from Cayman Chemical (Ann Arbor, MI, USA).

Primary antibodies for phospho-Akt (*p*-Akt; 1:1000, #9271), Akt (1:2000, #4691), phospho-p44/42 MAPK (*p*-ERK 1/2; 1:1000, #4370), p44/42 MAPK (ERK 1/2; 1:2000, #4695), catalase (1:1000, #12980), and SOD2 (1:2000, #13141) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin (1:20000, A1978) was a product of Sigma-Aldrich. For secondary antibodies, anti-rabbit (1:10000 or 1:20000, #7074) and anti-mouse (1:50000, NA931) were purchased from Cell Signaling Technology and GE Healthcare (Little Chalfont, UK).

2.2. Cell culture

Human lung adenocarcinoma A549 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (High Glucose DMEM; Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal bovine serum (BioWest, Nuaillé, France) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were seeded on 24-well plates (1.5×10^5 cells/well, for MTT assay) or 35-mm dishes (6.0×10^5 cells/dish, for western blotting). Reagents were added 24 h later. All treatment reagents contained 0.01% DMSO as a vehicle.

2.3. MTT assay

MTT assays were performed to evaluate cell viability. The MTT assay is a method for measuring aerobic succinate-MTT reductase activity in active mitochondria [18]. Therefore, it can be used to assess cytotoxicity, proliferation or activation [19]. We expressed cytotoxicity as cell viability.

MTT was dissolved in phosphate-buffered saline at 5 mg/mL and filtered through a 0.45-μm filter (Millex-HV; Merck Millipore, Darmstadt, Germany). After 24 h of drug treatment, 50 μL MTT solution was added to each well and the plate was incubated for 40 min at 37 °C. Then, the medium was discarded and 700 μL of DMSO was added to each well to dissolve stained cells. The absorbance was measured at a wavelength of 560 nm with background subtraction at 630 nm using a microplate reader (Multiskan JX; Thermo Labsystem, Helsinki, Finland) to calculate relative cell viability.

2.4. Western blotting

Cell samples were prepared as previously described [4] (with 140–200 μL of lysis buffer). Equal amounts of total protein (5 or 15 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gel (ATTO, Tokyo, Japan) and transferred onto a PVDF membrane (Immobilon-P; Merck Millipore). The membrane was sequentially treated with Block Ace (DS Pharma Biomedical, Osaka, Japan) and incubated overnight at 4 °C with primary antibodies in 10 mM Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 (TBST). The membrane was then washed with TBST and probed with secondary antibody in TBST for 1 h at room temperature. The washing procedure was repeated before the membrane was treated with a chemiluminescent reagent (ECL Prime, GE Healthcare). Protein expression was visualized using a LumiViewer EX140 (AISIN, Aichi, Japan) and quantified by LumiVision Analyzer140 (AISIN).

2.5. Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using the Tukey-Kramer's test. Significance was accepted when the *p*-value was less than 0.05.

3. Results

3.1. 4PBA and trolox attenuated PQ cytotoxicity

MTT assay was performed to assess the cytoprotective effect of 4PBA and trolox. Cell viability after treatment with 250 μM PQ for 24 h was reduced to approximately 70% compared with control in A549 cells. However, viability was recovered to approximately 90% by concomitant treatment of PQ with 5 mM 4PBA or 1 mM trolox (Fig. 1A and B). These results indicate that 4PBA and trolox significantly attenuated cell damage induced by PQ.

3.2. LY294002 inhibited the cytoprotective effect of 4PBA

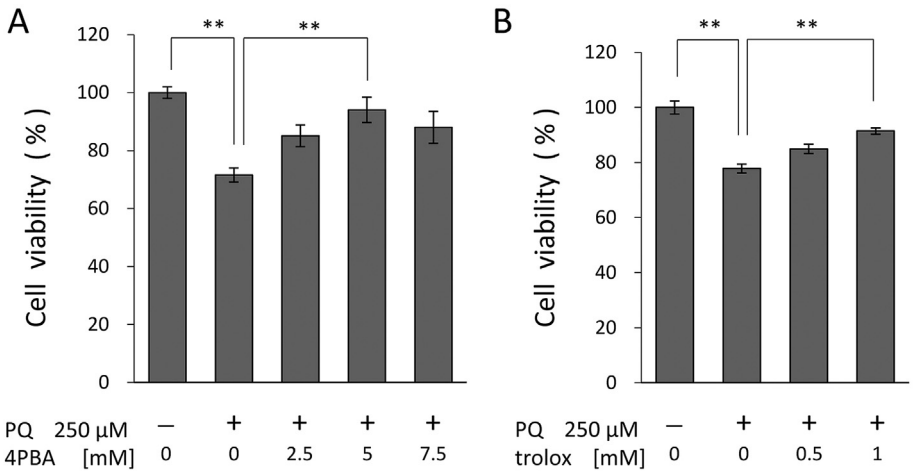
Involvement of major intracellular survival signaling pathways PI3K/Akt and MEK/ERK in the mechanism by which 4PBA and trolox elicit cytoprotective effects was investigated. Either 10 μM LY294002 (a PI3K inhibitor) or 10 μM U0126 (a MEK inhibitor) was incubated with PQ and 4PBA or trolox for 24 h before the MTT assay was performed. The cytoprotective effect of 4PBA on PQ toxicity was inhibited by co-treatment with LY294002. However, this phenomenon was not observed when cells were treated with U0126 (Fig. 1C). Both LY294002 and U0126 had no effect on cytoprotection by trolox (Fig. 1D).

3.3. 4PBA enhanced phosphorylation levels of ERK2 against PQ cytotoxicity

To confirm the involvement of PI3K/Akt and MEK/ERK pathways in the mechanism of 4PBA-elicited cytoprotection, phosphorylation levels of Akt and ERK were measured using western blotting and compared with trolox treatments. The phosphorylation level of ERK2 was increased 3 h after treatment with PQ and 4PBA, and was inhibited by LY294002 (Fig. 2B). The phosphorylation level of ERK1 was not increased. The phosphorylation level of Akt tended to increase, although there was no statistically significant difference (Fig. 2A). After 24 h of treatment, phosphorylation levels of ERK and Akt were not different between PQ only and PQ with 4PBA (Fig. 2C and D). Exposure to PQ and trolox did not affect those levels (Fig. 2E and F). Phosphorylation levels of Akt were increased in the presence of U0126 compared with the condition without inhibitors (Fig. 2A, C and E).

3.4. 4PBA induced up-regulation of catalase against PQ cytotoxicity

To examine the involvement of antioxidant enzymes in the mechanism of 4PBA and trolox cytoprotective effects, expression of catalase and SOD2 proteins were measured using western blotting. Catalase expression showed a tendency to decrease after PQ treatment (Fig. 3A, C and E). Co-treatment of PQ and 4PBA increased catalase expression after 24 h, however this up-regulation was not inhibited by either LY294002 or U0126 (Fig. 3C). SOD2 expression showed no statistically significant difference in response to 4PBA (Fig. 3B and D). Moreover, exposure to PQ and trolox did not affect those levels (Fig. 3E and F).


A
B
Cell viability (%)
120
100
80
60
40
20
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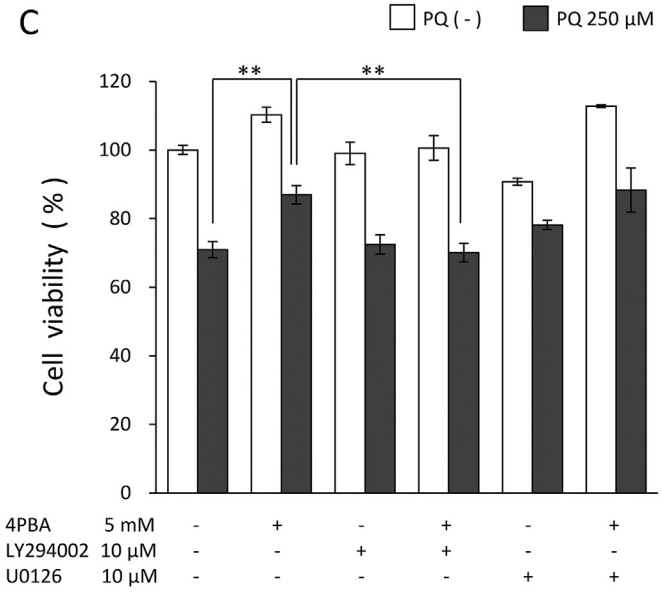
PQ 250 μM – + + + +
4PBA [mM] 0 0 2.5 5 7.5
PQ 250 μM – + + +
trolox [mM] 0 0 0.5 1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PQ (-)
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Cell viability (%)
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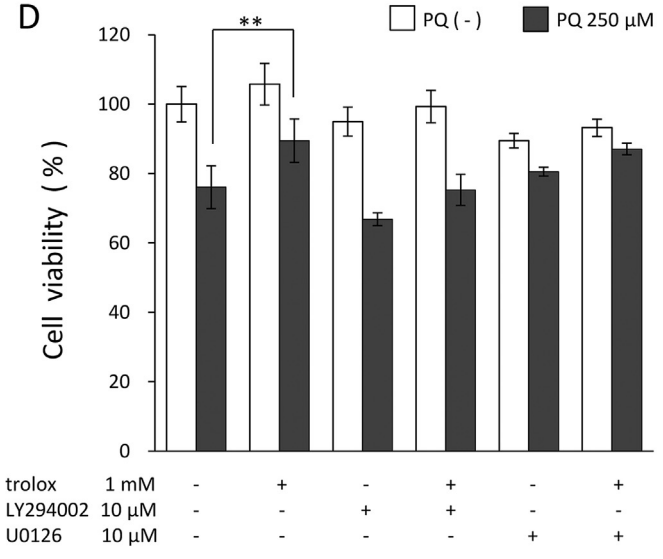
U0126 10 μM - - - - + +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PQ (-)
PQ 250 μM
Cell viability (%)
120
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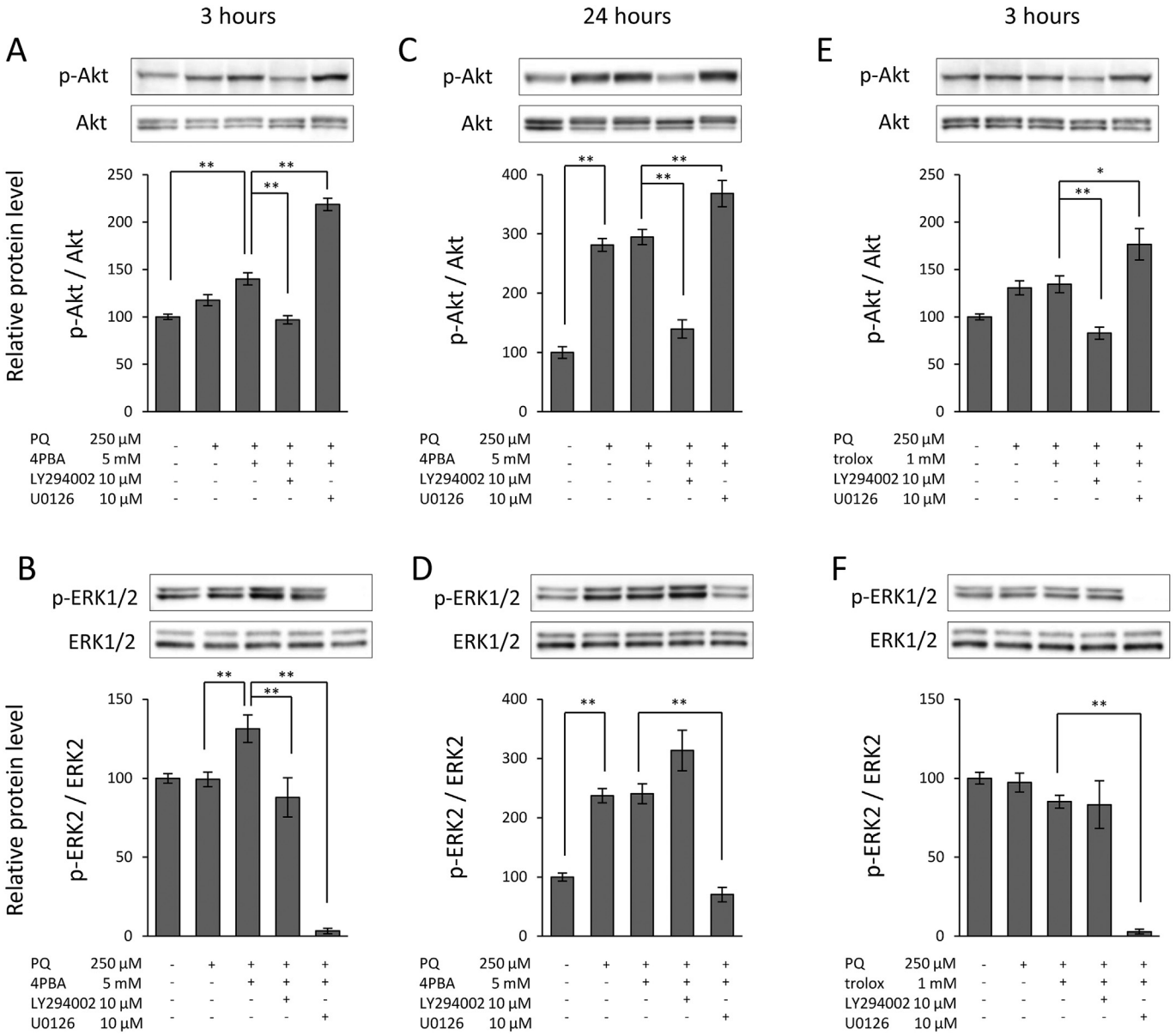

Fig. 2. Effects of 4PBA and trolox on Akt and ERK phosphorylation. A549 cells were treated with 5 mM 4PBA (A–D) or 1 mM trolox (E, F), with or without 10 μM LY294002 or 10 μM U0126 for 3 h (A, B, E, F) or 24 h (C, D) in the presence or absence of 250 μM PQ. Phosphorylation levels were detected by western blotting. Data are expressed as mean ± SEM of at least three independent experiments. Differences where p-values < 0.05 (*) and p-values < 0.01 (**) were significant by Tukey-Kramer's test.

4. Discussion

In this study, we demonstrated that both 4PBA and trolox attenuated PQ toxicity in A549 cells, but their mechanisms were different. 4PBA affected the intracellular survival signaling pathway MEK/ERK in the presence of PQ, while trolox did not show any effect.

4PBA increased ERK2 phosphorylation levels in the presence of PQ and this increase was inhibited by the PI3K inhibitor LY294002. The cytoprotective effect of 4PBA was completely inhibited by LY294002 according to the MTT assay. These results suggest that ERK2 phosphorylation plays a key role in 4PBA-induced cytoprotection via PI3K. This is consistent with a previous report indicating the involvement of PI3K in ERK phosphorylation [20].

Temporal regulation of ERK is an important factor in cellular responses [21]. The effect of ERK depends on whether activation is transient or persistent [22–24]. Increased ERK2 phosphorylation was observed 3 h after co-treatment with PQ and 4PBA compared with PQ treatment alone, but not after 24 h. In contrast, treatment with PQ only increased ERK phosphorylation for 24 h. Although

Fig. 1. Cytoprotective effects of 4PBA and trolox on PQ toxicity, and effects of inhibitors on 4PBA and trolox cytoprotection. (A, B) A549 cells were treated with indicated concentrations of 4PBA (A) or trolox (B) for 24 h with or without 250 μM PQ. Cell viability was evaluated by MTT assay. Data are expressed as mean ± SEM of four independent experiments. Differences where p-values < 0.01 (**) were significant by Tukey-Kramer's test. **(C, D)** A549 cells were treated with 5 mM 4PBA (C) or 1 mM trolox (D), with or without 10 μM LY294002 or 10 μM U0126 for 24 h in the presence (solid column) or absence (open column) of 250 μM PQ. Cell viability was calculated as percentage of control in the absence of PQ using an MTT assay. Data are expressed as mean ± SEM of at least three independent experiments. Differences where p-values < 0.01 (**) were significant by Tukey-Kramer's test when compared with the corresponding PQ-treated group.

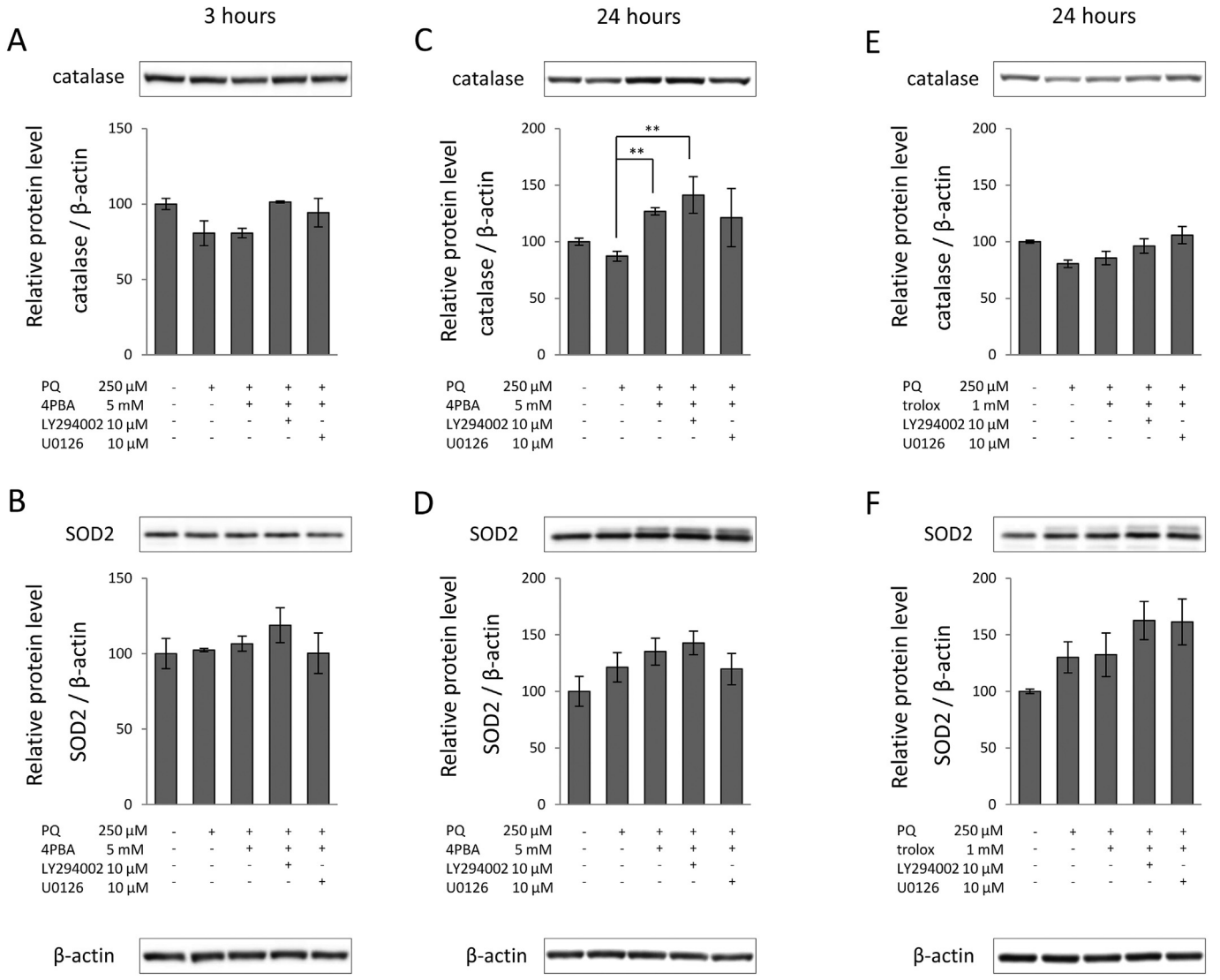

Fig. 3. Effects of 4PBA and trolox on catalase and SOD2 expression. A549 cells were treated with 5 mM 4PBA (A–D) or 1 mM trolox (E, F), with or without 10 μM LY294002 or 10 μM U0126 for 3 h (A, B) or 24 h (C–F) in the presence or absence of 250 μM PQ. Expression levels were detected by western blotting. Data are expressed as mean ± SEM of at least three independent experiments. Differences where p-values < 0.01 (**) were significant by Tukey-Kramer's test.

ERK is known as a MAP kinase that transduces survival signals [25], it also induces apoptosis [26]. ERK may be phosphorylated as a toxic mechanism of PQ [27,28]. Therefore, 4PBA is considered to phosphorylate ERK2 before activation by PQ. Our data suggest the possibility that 4PBA induced transient activation of ERK2 to attenuate PQ cytotoxicity.

Upon inhibition of ERK2 phosphorylation by the MEK inhibitor U0126, phosphorylation of Akt was significantly increased. Thus, we considered that when ERK activation was inhibited, phosphorylation of Akt compensated through cross-talk between MEK/ERK and PI3K/Akt pathways [29–31] to at least partially protect cells.

Expression of the antioxidant enzyme catalase was increased by 4PBA in the presence of PQ. Catalase, an enzyme that decomposes hydrogen peroxide into oxygen and water [32], is presumed to reduce oxidative stress caused by PQ. Although catalase expression levels did not decrease, the cytoprotective effect of 4PBA was decreased by LY294002. One of the other antioxidant enzyme, SOD2, which decomposes superoxide anions into hydrogen peroxide [32], was not increased by 4PBA. These results raise the possibility that an antioxidant effect may not be involved in the mechanism by which 4PBA protects cells against PQ toxicity. However, catalase gene expression is reportedly increased in cellular cytoprotective responses to PQ toxicity [33]. Thus, further investigation is required to confirm the relationship between antioxidant enzymes and the cytoprotective effect of 4PBA. Indeed, survival signal transduction and catalase up-regulation may be mediated by the chemical chaperone activity or histone deacetylase inhibitor activity of 4PBA, hence more research is necessary.

Trolox, an antioxidant derived from water-soluble vitamin E, is commonly used as a standard or positive control in antioxidant assays. Trolox acts as a reducing agent [34]. The mechanisms by which trolox induces antioxidant activity include reduction of reactive oxygen and nitrogen species produced by PQ radicals [2,3], and trolox attenuates PQ cytotoxicity (Fig. 4).

In conclusion, our study suggests that 4PBA enhances phosphorylation of ERK2 via PI3K to attenuate PQ toxicity in A549 cells. 4PBA also increases catalase expression, although this increase may not be involved in enhanced cell viability. These results may provide new findings for understanding the molecular mechanism by

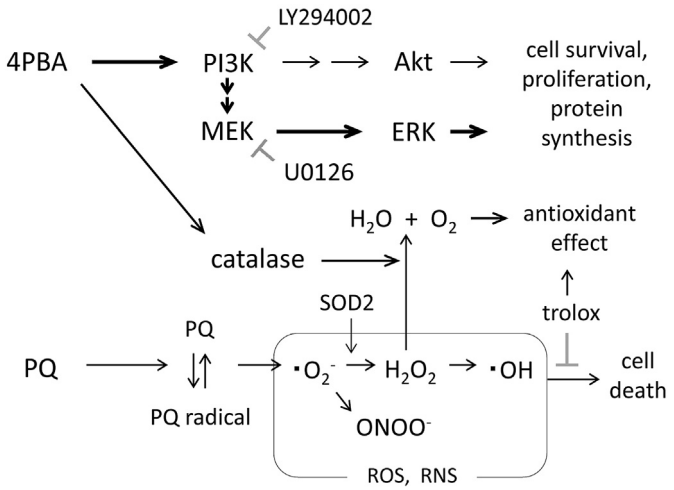

Fig. 4. Potential site of 4PBA action in attenuating PQ cytotoxicity. 4PBA attenuates PQ cytotoxicity through ERK2 activation via PI3K. When ERK2 phosphorylation is inhibited by the MEK inhibitor U0126, Akt is phosphorylated in a compensatory manner and PQ toxicity is attenuated. When PI3K is inhibited by LY294002, both ERK2 and Akt are not phosphorylated and the cytoprotective effect is lost. ROS; reactive oxygen species, RNS; reactive nitrogen species.

which 4PBA elicits cytoprotection, as well as new therapeutic targets for PQ toxicity.

Conflicts of interest

We have no financial relationships to disclose.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.06.080.

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