Negative regulation of ERK1/2 by PI3K is required for the protective effects of *Pyropia yezoensis* peptide against perfluorooctane sulfonate-induced endoplasmic reticulum stress

JEONG HWAN OH[1], EUN-YOUNG KIM[1], YOUN HEE CHOI[2] and TAEK-JEONG NAM[1,3]

[1]Cell Biology Laboratory, Institute of Fisheries Sciences, Pukyong National University, Busan 46041; Departments of [2]Marine Bio-Materials and Aquaculture, and [3]FoodScience and Nutrition, Pukyong National University, Busan 48513, Republic of Korea

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Abstract. Perfluorooctane sulfonate (PFOS) is a stable fluorosurfactant, which causes adverse effects in various organisms. The present study was designed to investigate the effects of *Pyropia yezoensis* peptide (PYP), a peptide comprised of 11 residues (ALEGGKSSGGG), on PFOS-induced endoplasmic reticulum (ER) stress in Chang cells. PFOS exposure (400 μM) for 24 h significantly decreased cell viability, which was upregulated by 250-1,000 pg/ml PYP treatment. Exposure to PFOS also significantly increased expression of the ER stress response protein, glucose-regulated protein 78 (GRP78), and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). These elevations were significantly decreased by PYP (250 pg/ml), and, in particular, the PFOS-induced GRP78 upregulation was decreased following treatment with 10 μM SL327, an ERK-kinase inhibitor. However, PYP-induced decreases in GRP78 expression and ERK1/2 phosphorylation were upregulated following treatment with LY294002 (20 μM), a phosphatidylinositol-3 kinase (PI3K) inhibitor. PFOS-induced apoptosis was also significantly attenuated by PYP (250 pg/ml) treatment, and the PYP-induced reduction in apoptosis was abolished by inhibition of PI3K. These findings indicate that negative regulation of ERK1/2 by PI3K is essential for the protective effects of PYP against PFOS-induced cell death, suggesting that PYP may be a candidate for therapeutic use.

Introduction

Perfluorooctane sulfonate (PFOS) is an organofluorine compound and a synthetic, stable fluorosurfactant that is used as a surface protector for paper, food containers, carpets and various other applications due to its hydrophobic and lipophobic properties (1). Fluorine has the highest electronegativity in fluorocarbons, resulting in formation of a strong carbon-fluorine (C-F) covalent bond, thus inducing resistance to hydrolysis, photolysis and biodegradation. Therefore, fluorocarbons are considered persistent organic pollutants, and pharmacokinetic studies on PFOS have been conducted in fish, monkeys, chickens and humans (2-4). These studies revealed that PFOS has a relatively long depuration half-life, which may disturb cellular function. Although the mechanisms underlying the toxicity of PFOS have not been fully established, the chemical is known to induce oxidative stress and cellular damage, including hepatocellular hypertrophy and the inhibition of intracellular communication (5,6).

The endoplasmic reticulum (ER) is a major organelle that is involved in protein modification and folding, as well as intracellular calcium homeostasis. Cellular stress-induced protein damage and alteration of redox status results in a reduction of folding capacity and the accumulation of misfolded proteins in the ER lumen, which activates a series of signaling pathways known as the ER stress response (7,8). Glucose-regulated protein 78 (GRP78), which is an ER stress sensor, is an ATP-dependent protein chaperone localized in the ER lumen. Under ER stress, GRP78 binds unfolded proteins and activates a multi-chaperone complex, resulting in increased ER protein folding capacity (9). However, severe and long-lasting ER stress results in the accumulation of unfolded or misfolded proteins and subsequent cell death.

Pyropia yezoensis is a red alga that has been cultured as food and a nutritional supplement due to its biofunctional components, including proteins, vitamins, minerals and mycosporine-like amino acids (10). In particular, *P. yezoensis* peptide (PYP) is known to have antioxidant and chemoprotective properties (11,12). However, the bioactivity of PYP in ER stress conditions induced by environmental pollutants has yet to be elucidated.

The present study was designed to investigate the hypothesis that the protective effects of PYP against PFOS exposure are associated with the ER stress response, and that this is mediated by the phosphatidylinositol-3 kinase (PI3K) and

Correspondence to: Professor Taek-Jeong Nam, Cell Biology Laboratory, Institute of Fisheries Sciences, Pukyong National University, 474 Ilgwang-ro, Ilgwang-myeon, Gijang-gun, Busan 46041, Republic of Korea
E-mail: namtj@pknu.ac.kr

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extracellular signal-regulated kinase 1/2 (ERK1/2) signal pathways. To investigate this hypothesis, it was determined whether i) pretreatment with PYP decreases ER stress caused by PFOS exposure; ii) the PYP-induced decrease in PFOS-induced ER stress is associated with the PI3K and ERK1/2 signaling pathways, and iii) apoptosis induced by PFOS exposure is regulated by PYP-induced activation of the PI3K signaling pathway.

Materials and methods

Cell culture and chemicals. Chang cells were purchased from American Type Culture Collection (Manassas, VA, USA; cat. no. CCL-13). This cells line is known to have been contaminated with HeLa cervical adenocarcinoma cells. The cells were cultured in minimum essential medium containing non-essential amino acids (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 1 mM sodium pyruvate, 10% fetal bovine serum (GenDEPOT, Inc., Barker, TX, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a humidified incubator containing 5% CO_2. PFOS (cat. no. 2795-39-3; >98%) and dimethyl sulfoxide (DMSO; cat. no. 67-68-5; >99.9%) were purchased from Sigma-Aldrich; Merck KGaA, and LY294002 (cat. no. 1130) and SL327 (cat. no. 1969) were obtained from Tocris Bioscience (Bristol, UK). PFOS and inhibitors were dissolved in DMSO. The minimal concentration of DMSO (<0.001%) was used to prevent cellular damage.

Cell viability assay. Cell viability was determined using Cyto-X™ cell viability assay kit (LPS Solution, Daejeon, South Korea). Cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate (final volume, 100 μl/well), and were incubated for 24 h at 37˚C in a humidified incubator containing 5% CO_2. Cells were then exposed to PFOS (25-400 μM) for 24 h, with or without pretreatment with PYP (62.5, 125, 250, 500 or 1,000 pg/ml) for 2 h at 37˚C. PYP is a peptide comprising 11 residues (ALEGGKSSGGG), which was synthesized by Peptron (Daejeon, South Korea) according to a previous study (12). Subsequently, a water-soluble tetrazolium salt (10 μl/well) was added and the cells were incubated for 30 min at 37˚C in a 5% CO_2 incubator. Colored formazan was measured by examining the absorbance at 450 nm.

Western blotting. PFOS-induced ER stress was confirmed using immunoblotting. Cells were treated with PFOS (400 μM) for 24 h at 37˚C, with or without PYP (250 pg/ml) pretreatment for 2 h at 37˚C. Additionally, 1 group was treated with PYP (250 pg/ml) only. SL327 (10 μM) and LY294002 (20 μM) treatment was performed for 30 min prior to the PYP pretreatment. Following treatment, the media was removed and cells were washed twice with 1X PBS. Cell lysis was performed with radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Calbiochem; Merck KGaA). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and proteins (20 μg) were separated by 15% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with a blocking buffer containing 1% bovine serum albumin (GenDEPOT, Inc.) in TBST (TBS containing 0.1% Tween-20) and was then probed with the following primary antibodies: Anti-GRP78 (1:1,000; cat. no. ab108613; Abcam, Cambridge, UK), anti-pERK1/2 (1:1,000; cat. no. sc-7383; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), ERK1 (1:1,000; cat. no. sc-94; Santa Cruz Biotechnology, Inc.), ERK2 (1:1,000; cat. no. sc-154; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.) overnight at 4˚C with agitation. Following washing three times with TBST for 10 min, the membrane was incubated with the appropriate secondary antibody (cat. no. 31460; Thermo Fisher Scientific, Inc.) at a dilution of 1:10,000 for 1 h at room temperature. b-actin was probed for the normalization of blots.

Detection of apoptotic cells. Cells were treated with 400 μM PFOS for 24 h at 37˚C, with or without PYP pretreatment (250 pg/ml) for 2 h at 37˚C. Apoptosis was measured using the Muse Annexin V and Dead Cell Assay kit (Merck KGaA) according to the manufacturer's protocol. Annexin V staining was measured by Muse® Cell Analyzer and the data was analysed by Muse 1.3.1 software (Merck KGaA).

Statistical analysis. All experiments were performed in triplicate and the data were expressed as the mean ± standard error of the mean for each group. For western blotting, the difference in expression levels was determined by densitometric analysis, using ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA). Data were statistically analyzed by one-way analysis of variance followed by Tukey's multiple comparison tests using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PYP attenuates PFOS-induced decreases in cell viability and downregulates PFOS-induced increases in GRP78 expression in Chang cells. To investigate the effects of PYP on PFOS-induced cell damage, the optimal dose of PFOS was initially determined based on a cell viability assay. Exposure to PFOS (100 and 400 μM) significantly decreased the viability of Chang cells compared with the untreated control cells (Fig. 1A). Following exposure to 400 μM PFOS for 24 h with or without PYP pretreatment (62.5-1,000 pg/ml), cell viability was assessed in a parallel manner. Pretreatment with PYP (250-1,000 pg/ml) significantly attenuated the PFOS-induced decrease in cell viability compared with in cells exposed to PFOS alone (Fig. 1B). To further investigate whether the PYP-induced increase in cell viability was associated with the ER stress response, cell damage was induced by 400 μM PFOS in order to decrease the viability of Chang cells by ~50%. Subsequently, ER stress was assessed by measuring the expression levels of GRP78 by western blotting. GRP78 expression was upregulated following exposure to PFOS for 24 h compared with the control cells; however, GRP78 expression was significantly downregulated by pretreatment with PYP (250 pg/ml; Fig. 1C). There was no significant alteration in GRP78 expression between the control group and the group treated with PYP alone (250 pg/ml; Fig. 1C).

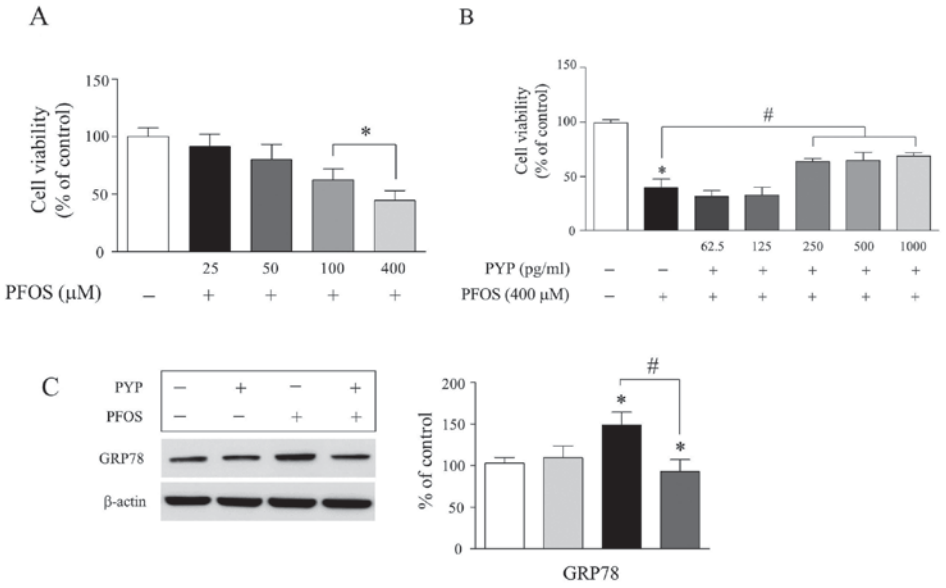

Figure 1. Effects of PYP treatment on cell viability and PFOS-induced ER stress. (A) PFOS decreased the viability of Chang cells in a dose-dependent manner. (B) Pretreatment with PYP (250-1,000 pg/ml) significantly increased the viability of PFOS-treated cells. (C) Exposure to PFOS (400 μM) significantly upregulated GRP78 expression, which was attenuated by pretreatment with PYP (250 pg/ml). *P<0.05 vs. control group; #P<0.05 vs. PFOS (400 μM) exposure. PYP, *Pyropia yezoensis* peptide; ER, endoplasmic reticulum; PFOS, perfluorooctane sulfonate; GRP78, glucose-regulated protein 78.

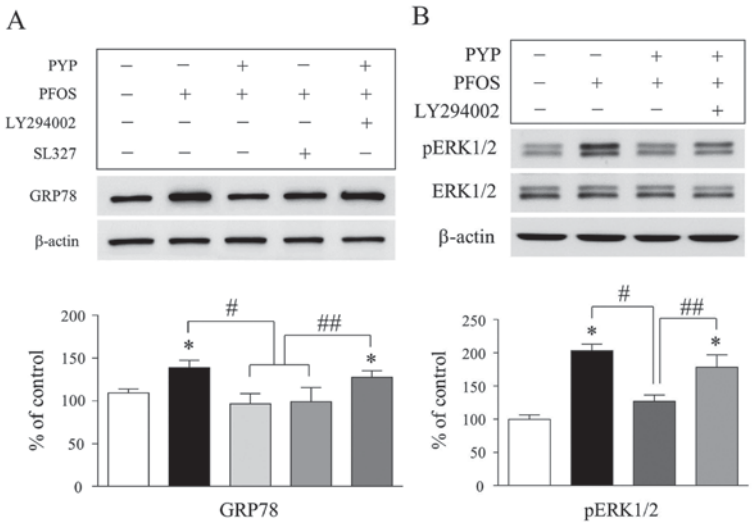

Figure 2. PYP induced activation of PI3K-dependent signaling and decreased ERK1/2-mediated ER stress in response to PFOS exposure. (A) PFOS-induced increase in GRP78 expression was significantly downregulated by pretreatment with SL327 (10 μM). Inhibition of PI3K with LY294002 (20 μM) attenuated the 250 pg/ml PYP- and SL327-induced decrease in GRP78 expression. (B) Phosphorylation of ERK1/2 by PFOS (400 μM) was decreased by PYP (250 pg/ml) pretreatment, which was upregulated by inhibition of PI3K. *P<0.05 vs. control group; #P<0.05 vs. PFOS (400 μM) exposure; ##P<0.05 vs. pretreatment with PYP or SL327. PYP, *Pyropia yezoensis* peptide; PI3K, phosphatidylinositol-3 kinase; ERK1/2, extracellular signal-regulated kinase 1/2; ER, endoplasmic reticulum; PFOS, perfluorooctane sulfonate; GRP78, glucose-regulated protein 78.

Inhibition of PI3K attenuates PYP-induced decreases in GRP78 expression and ERK1/2 phosphorylation by PFOS. To investigate the intracellular events modulated by PYP against PFOS-induced ER stress, the present study aimed to determine i) whether PFOS-induced GRP78 expression was associated with the PI3K and ERK1/2 signal pathways, and ii) whether there was an interaction between PI3K and ERK1/2 for PYP-induced decreases in PFOS-induced ER stress. Pretreatment with SL327 (10 μM), an ERK-kinase inhibitor, significantly downregulated PFOS-induced GRP78 expression compared with cells exposed to PFOS alone. Conversely, pretreatment with LY294002 (20 μM), an inhibitor of PI3K, upregulated 250 pg/ml PYP-inhibited GRP78 expression compared with cells exposed to PFOS and pretreated with PYP (Fig. 2A). Since activation of PI3K-dependent signalling (as inhibition of PI3K attenuated the PYP-induced decrease in PFOS-induced ER stress) and inhibition of ERK1/2 phosphorylation were associated with PYP-induced decreases in PFOS-induced ER stress, the present study aimed to determine whether PYP-induced activation of PI3K regulated phosphorylation of ERK1/2. Phosphorylation of ERK1/2 by PFOS was significantly

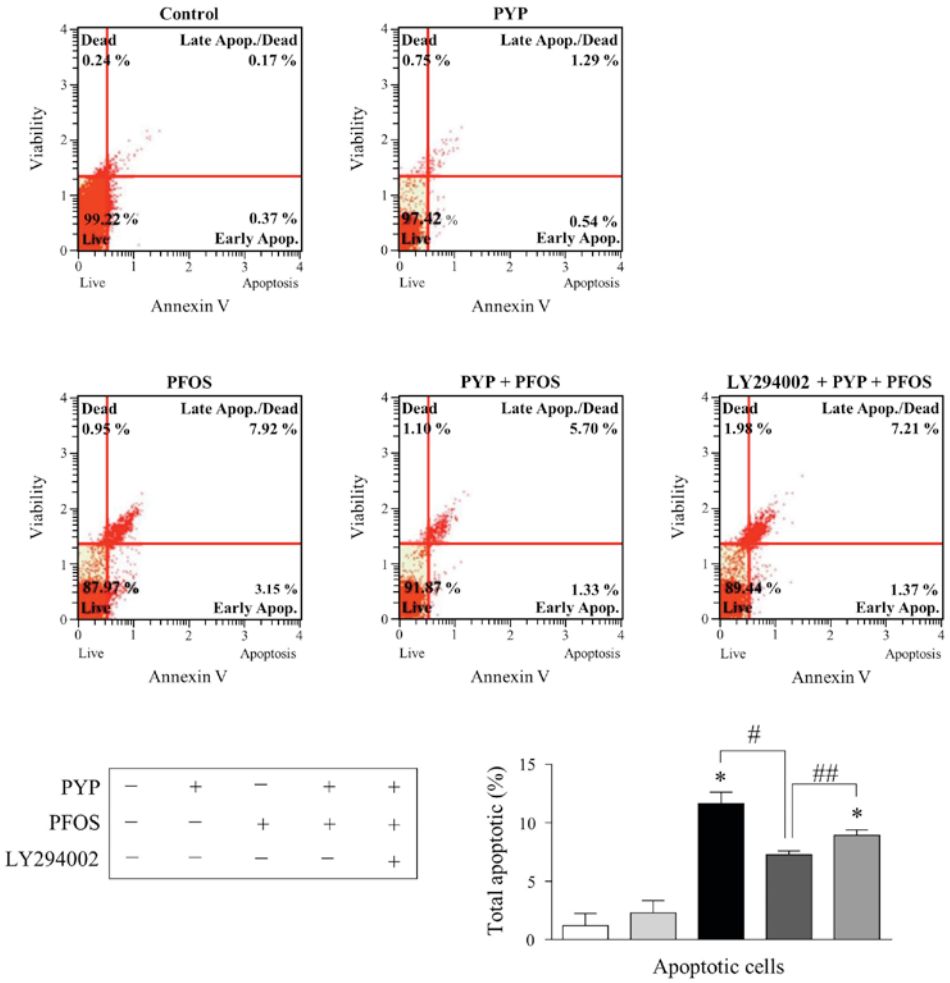

Figure 3. Protective effects of PYP against PFOS-induced cell death. Exposure to PFOS (400 μM) increased the rate of apoptosis, which was significantly attenuated by pretreatment with PYP (250 pg/ml). The PYP-induced decrease in apoptosis was attenuated by inhibition of phosphatidylinositol-3 kinase with LY294002 (20 μM). *P<0.05 vs. control group; $^{\#}$P<0.05 vs. PFOS (400 μM) exposure; $^{\#\#}$P<0.05 vs. LY294002 pretreatment. PYP, *Pyropia yezoensis* peptide; PFOS, perfluorooctane sulfonate.

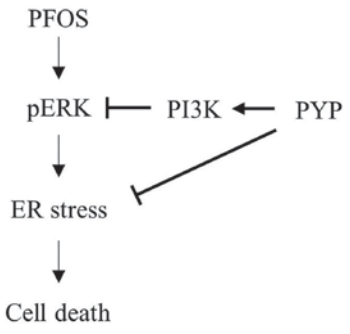

Figure 4. Schematic of a proposed mechanism underlying the protective effects of PYP against PFOS exposure in Chang cells. PFOS-induced ER stress is mediated by phosphorylation of ERK1/2. The phosphorylation of ERK1/2 and ER stress are downregulated by PYP-induced activation of PI3K. PYP increases the viability of Chang cells via a decrease in PFOS-induced ER stress by PI3K activation, attenuating PFOS-induced apoptosis. PYP, *Pyropia yezoensis* peptide; PFOS, perfluorooctane sulfonate; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1/2; PI3K, phosphatidylinositol-3 kinase.

decreased by PYP (250 pg/ml) treatment, which was abolished by inhibition of PI3K with LY294002 (20 μM; Fig. 2B). There was no significant difference in the total ERK1/2 expression compared with the control (Fig. 2B).

PFOS-induced cell death was decreased by activation of PI3K by PYP treatment. Since PYP treatment enhanced cell viability and decreased PFOS-induced ER stress, it was determined whether PFOS-induced apoptosis was regulated by PYP treatment. The number of apoptotic cells was significantly increased by PFOS (400 μM) exposure for 24 h compared with the control cells (Fig. 3). Pretreatment with PYP (250 pg/ml) significantly decreased PFOS-induced apoptosis; however, this effect was attenuated by inhibition of PI3K with LY294002 (20 μM; Fig. 3A). There was no significant difference between the PYP and control groups.

Discussion

The present study was performed to investigate the protective effects of PYP against PFOS-induced ER stress in Chang cells. PYP (250 pg/ml) treatment downregulated the PFOS-induced increase in ER stress, mediated by phosphorylation of ERK1/2. The PYP-induced decrease in ER stress was reversed by inhibition of PI3K. In addition, PFOS-induced

apoptosis was significantly decreased by PYP treatment, which was abolished by inhibition of PI3K. Therefore, these findings demonstrated that the protective effects of PYP occur via inhibition of ERK1/2, resulting in the activation of PI3K against PFOS-induced ER stress.

Persistent organic pollutants, such as PFOS, induce oxidative stress, which induces apoptosis and developmental and reproductive toxicity in rats and freshwater fish. In particular, PFOS-induced oxidative stress results in ER stress and affects intracellular signaling, which subsequently leads to cell death (13-17). In the present study, it was demonstrated that pretreatment with PYP (250-1,000 pg/ml) enhanced the viability of Chang cells against PFOS (400 μM) exposure, and PYP (250 pg/ml) treatment significantly decreased ER stress by PFOS (400 μM) (Fig. 1). These results suggested that PYP may protect Chang cells against the ER stress caused by PFOS exposure.

To further explore the mechanisms underlying the inhibitory effects of PYP on PFOS-induced ER stress, PI3K and ERK1/2 signal pathways were assessed by western blotting. PI3K and ERK signaling pathways are known to be involved in controlling cell survival and proliferation in response to various extracellular stimuli (18,19). However, PI3K and ERK1/2 inhibit each other and negatively regulate the other's pathway (20,21). Although ERK1/2 has been demonstrated to promote cell survival, activation of ERK1/2 also induces apoptosis depending on the cell type examined (22,23). In the present study, the PFOS-induced increase in GRP78 expression was downregulated by PYP (250 pg/ml) treatment, as well as following inhibition of ERK1/2 with SL327 (10 μM). However, the PYP-induced decrease in GRP78 expression was upregulated upon inhibition of PI3K with LY294002 (20 μM; Fig. 2A). Phosphorylation of ERK1/2 by PFOS (400 μM) exposure was also decreased by PYP (250 pg/ml) treatment, which was significantly upregulated by inhibition of PI3K (Fig. 2B). In addition, PFOS-induced apoptosis was significantly decreased by PYP (250 pg/ml) treatment, and was abolished following inhibition of PI3K (Fig. 3).

In conclusion, these results demonstrated that i) PFOS-induced ER stress is mediated by phosphorylation of ERK1/2; ii) ERK1/2 phosphorylation is negatively regulated by PYP-induced activation of PI3K; and iii) inhibition of PI3K reverses the PYP-induced decrease in PFOS-induced ER stress and apoptosis (Fig. 4). These findings suggested that negative regulation of ERK1/2 by PI3K is essential for the protective effects of PYP against PFOS-induced ER stress-induced apoptosis of Chang cells. Thus, PYP may be used to decrease the health risks caused by environmental pollutants.

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