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Involvement of MEK–ERK signaling pathway in the inhibition of cell growth by troglitazone in human pancreatic cancer cells

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Abstract

In the present study, we examined a role of mitogen-activated protein kinases (MAPKs), extracellular signal-related kinase (ERK), c-Jun N-terminal protein kinase, and p38 MAPK in troglitazone-induced inhibition of cell growth in human pancreatic cancer cells. Among the three kinases, troglitazone specifically inhibited the phosphorylation of ERK1/2 in a dose- and time-dependent manner. Troglitazone also down-regulated the protein expression of mitogen-activated protein kinase kinase (MEK)1/2, an upstream molecule that regulates ERK phosphorylation. Treatment of human pancreatic cancer cells with specific MEK inhibitor, PD98059 or U0126, inhibited ERK1/2 phosphorylation and cell growth. These results suggest for the first time that the inhibition of the MEK1/2–ERK1/2 signaling pathway may be implicated in the growth inhibitory effect by troglitazone in human pancreatic cancer cells.

Keywords: Troglitazone; Extracellular signal-related kinase; Mitogen-activated protein kinase kinase; Pancreatic cancer cells; Growth inhibition; PPARγ

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid [1]. PPARγ is expressed at high levels in adipose tissue and functions as a key molecule of adipocyte differentiation [2,3]. In addition to adipose tissue, PPARγ expression is detected in a wide variety of tumor cells [4–13]. In the tumor cells, PPARγ activation by its high affinity ligands could inhibit cell growth and induce apoptosis. Thus, PPARγ is involved not only in lipid metabolism but also cellular proliferation in cancer cells. It is therefore suggested that PPARγ is considered as a possible molecular target for cancer treatment.

With regard to the mechanism by which PPARγ activation by its ligands induces growth arrest and apoptosis, we have already demonstrated that protein accumulation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor (CDKI) [14,15], and p53 plays a role in the growth arrest and apoptosis in human pancreatic and gastric cancer cells [16–18]. However, little is known whether other molecules that regulate cell proliferation and apoptosis are involved in the cellular behavior evoked by PPARγ ligands such as troglitazone.

Mitogen-activated protein kinases (MAPKs) are essential components of the intracellular signal transduction pathways that regulate cell proliferation and apoptosis [19–21]. There are three subgroups of MAPKs, extracellular signal-related kinases (ERKs), c-Jun N-terminal protein kinases (JNKs), and p38 MAPKs. For instance, activation of ERK culminates in phosphorylation of transcription factors responsible for regulating genes that enhance cell proliferation and protect cells from apoptosis [22,23]. In the present study,

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we examined the hypothesis that MAPKs may be involved in the mechanism by which troglitazone inhibits cell growth in human pancreatic cancer cells.

Materials and methods

Cell culture. Human pancreatic cancer cell line, PK-1 [24], was obtained from Tohoku University, Sendai, Japan. Our previous study confirmed that PK-1 cells express PPARγ [16]. The pancreatic cancer cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin, and 10% fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Chemicals. Troglitazone, a selective ligand for PPARγ, [25] was kindly provided from Sankyo Pharmaceutical (Tokyo, Japan) and was dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 0.05% in the culture medium. MEK inhibitors, U0126 and PD98059 [26,27], were purchased from Promega Biosciences, Madison, WI, USA.

Western blotting analysis. Total protein was extracted from PK-1 cells. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA) following the manufacturer's suggested procedure. Fifty micrograms of protein was separated by 10% SDS–PAGE (PAG Mini DAIICHI, Daiichi Pure Chemicals, Tokyo, Japan). After electrophoresis, the proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA), blocked overnight in Block Ace (Dainippon Seiyaku, Osaka, Japan) at 4 °C, reacted with primary polyclonal antibody against human actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), P44/42 MAP kinase antibody, phospho-P44/42 MAPK (Thr202/Tyr204) antibody, p38 MAP kinase antibody, phospho-p38 MAP kinase (Thr180/Tyr182) antibody, SAPK/JNK antibody, phospho-SAPK/JNK(Thr183/Tyr185) antibody, MEK1/2 antibody or phospho-MEK1/2(Ser217/221) antibody (Cell Signaling Technology, Beverly, MA, USA) for 1 h, washed with TBS-T, reacted with secondary polyclonal antibody against rabbit IgG (Chemicon International, Temecula, CA, USA) for 1 h, and washed with TBS-T. After reaction with horseradish peroxidase-conjugated anti-rabbit IgG immune complexes were visualized by using the ECL plus detection reagents (Amersham International, NJ, USA) following the manufacturer's suggested procedure. Normal rabbit IgG was used simultaneously as a control.

Cell growth assay. Cell number was evaluated by WST-1 assay according to our previous reports [16,28]. Cells were seeded on a 96-well cell culture cluster (Corning, Corning, NY, USA) at a concentration of 2×10^4/well in a volume of 100 μl. Twenty-four hours later, each well was incubated with troglitazone, U0126 or PD98059 at several concentrations for 0 or 48 h. Cell numbers were measured colorimetrically using the Cell Counting Kit (Dojindo, Kumamoto, Japan) by ImmunoMini NJ-2300 (NJ InterMed, Tokyo, Japan) at a test wavelength of 450 nm. This assay is based on the cleavage of the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) by mitochondrial dehydrogenase in viable cells [29].

Statistical analysis. The results are expressed as means ± SEM. Statistical analysis was performed by repeated measures ANOVA and subsequent Fisher's LSD test. A value of $P < 0.05$ was considered statistically significant.

Results

Troglitazone inhibits ERK1/2 phosphorylation in human pancreatic cancer cells

Fig. 1 shows that 24 h after treatment of troglitazone, inhibition of ERK1/2 phosphorylation was observed

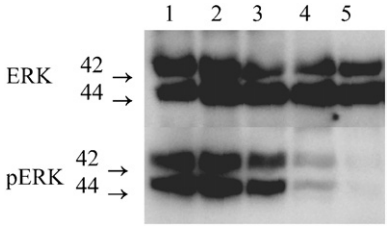

Fig. 1. Dose–response effect of troglitazone on total and phosphorylated ERK1/2 protein expression in human pancreatic cancer cells. PK-1 cells were treated with several doses of troglitazone and 24 h after treatment, total and phosphorylated ERK1/2 protein expression was detected by Western blot. Western blot for total or phosphorylated ERK1/2 in PK-1 cells treated with each dose of troglitazone is shown in lane 1 (DMSO control), lane 2 (0.1 μM troglitazone), lane 3 (1 μM troglitazone), lane 4 (10 μM troglitazone) or lane 5 (100 μM troglitazone), respectively.

while troglitazone failed to change the total amount of ERK1/2, as determined by Western blotting. A drastic inhibition of ERK1/2 phosphorylation was observed by the doses of over 10 μM troglitazone and the inhibition was dose-dependent. In addition to ERK1/2, we also examined the phosphorylation state of two other MAP kinases, JNK and p38 MAPK, by Western blotting with phospho-specific antibodies. No consistent changes in the phosphorylation of JNK or p38 MAPK were detected after treatment with troglitazone (data not shown).

Troglitazone inhibits MEK1/2 protein expression in human pancreatic cancer cells

The only characterized upstream activator of ERK1/2 is MEK1/2 [19]. We, therefore, determined whether troglitazone inhibits ERK1/2 phosphorylation by inhibiting its upstream activator, MEK1/2. Like ERK1/2, MEK1/2 is activated by phosphorylation, and phospho-specific antibodies were used to assay MEK1/2 activity by Western blotting. As shown in Fig. 2A, troglitazone inhibited MEK1/2 phosphorylation with a time and dose dependence similar to that of the inhibition of phosphorylated ERK1/2, which indicated that troglitazone inhibits ERK1/2 phosphorylation by acting upstream of, and not at the level of, ERK1/2. Unlike total ERK1/2 expression, total MEK1/2 expression, as determined by Western blotting with pan-MEK1/2 antibody, was decreased after treatment with troglitazone. Total MEK1/2 protein expression was considerably inhibited by troglitazone throughout the period observed from 12 to 48 h (Fig. 2B).

U0126 and PD98059 induce growth arrest in human pancreatic cancer cells

To clarify whether inhibition of ERK activity alone could inhibit cell growth in pancreatic cancer cells,

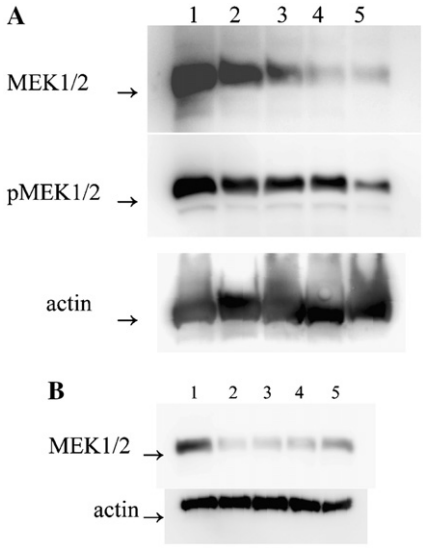

Fig. 2. (A) Dose–response effect of troglitazone on total and phosphorylated MEK1/2 protein expression in human pancreatic cancer cells. PK-1 cells were treated with several doses of troglitazone and 48 h after treatment, total and phosphorylated MEK1/2 protein expression was detected by Western blot. Western blot for total (MEK1/2) or phosphorylated MEK1/2 (pMEK1/2) in PK-1 cells treated with each dose of troglitazone is shown in lane 1 (DMSO control), lane 2 (0.1 μM troglitazone), lane 3 (1 μM troglitazone), lane 4 (10 μM troglitazone) or lane 5 (100 μM troglitazone), respectively. (B) Time course effect of troglitazone on total MEK1/2 protein expression in human pancreatic cancer cells. Each lane represents the result of cells 0 (lane 1), 12 (lane 2), 24 (lane 3), 36 (lane 4) or 48 (lane 5) h after troglitazone treatment.

effects of MEK inhibitors on cell growth were evaluated. Fig. 3 demonstrates the effect of troglitazone or MEK inhibitors, U0126 and PD98059, on ERK1/2 phosphorylation in human pancreatic cancer cells. Troglitazone at a dose of 100 μM potently suppressed ERK phosphorylation at 24 and 48 h. It was also shown that both MEK inhibitors, U0126 and PD98059, completely blocked ERK1/2 phosphorylation throughout the period observed, indicating that both the chemicals block ERK activities. Using the inhibitors, WST-1 assay was performed to evaluate the effect of the chemicals on cell

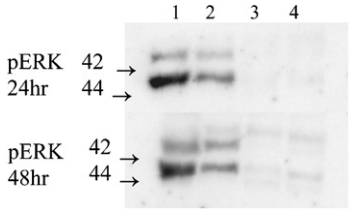

Fig. 3. Phosphorylated ERK1/2 expression by troglitazone, U0126 or PD98059 in human pancreatic cancer cells. Phosphorylated ERK1/2 (pERK) protein expression was detected by Western blot in PK-1 cells treated with troglitazone, U0126 or PD98059 at 24 h (upper panel) and 48 h (lower panel). Each lane represents the result of DMSO control (lane 1), 100 μM troglitazone (lane 2), 50 μM U0126 (lane 3) or 50 μM PD98059 (lane 4), respectively.

growth. As illustrated in Fig. 4, troglitazone dose-dependently inhibited cell growth, being in good agreement with our previous report [16]. It was also demonstrated that U0126 and PD98059 inhibited cell growth in a dose-dependent fashion.

Discussion

We have already demonstrated that human pancreatic cancer cells express functional PPARγ and PPARγ activation by its specific ligands such as troglitazone-induced cell growth arrest and inhibited cell invasion [16,30]. These results suggest that PPARγ is a possible molecular target for pancreatic cancer treatment and PPARγ ligands such as thiazolidinediones may be relevant for cancer therapy. There is however a little evidence about the molecular mechanisms by which troglitazone exerts its anti-proliferative action. Along this line, we have demonstrated that $p27^{Kip1}$ and p53 are implicated in the cytotoxicity by troglitazone in human pancreatic and gastric cancer cells [16–18,28]. The present study was performed to try to clarify the role of MAPKs in cell growth inhibition by a PPARγ ligand, troglitazone, in human pancreatic cancer cells. Among three MAPKs, ERK1/2, JNK, and p38 MAPK, troglitazone specifically suppressed ERK1/2 phosphorylation in human pancreatic cancer cells. It has been reported that inhibition of ERK phosphorylation induces cell growth arrest in a couple of cancer cells. For instance, inhibition of ERK1/2 phosphorylation in vitro by a synthetic MEK1/2 inhibitor, PD184352, decreased soft agar growth and inhibited the transformed phenotype of colon 26 cells. In vivo, PD184352 suppressed the growth of mouse and human colon tumor xenografts [31]. Rice et al. [32] have demonstrated that U0126, the specific MEK inhibitor, resulted in an inhibition of ERK phosphorylation and cytotoxicity in cultured colon cancer cells. Based on these evidences, we would suggest that inhibition of ERK1/2 phosphorylation might be involved in the cell growth inhibition by troglitazone. Inhibition of ERK1/2 phosphorylation and cell growth was observed by the same doses (over 10 μM) of troglitazone shown in Figs. 1 and 4, furthermore supporting the above speculation.

Next, we examined the possible mechanism by which troglitazone inhibits ERK1/2 phosphorylation. Because the only characterized upstream activator of ERK1/2 is MEK1/2 [19], we determined whether troglitazone inhibits ERK1/2 phosphorylation by inhibiting its upstream activator, MEK1/2. The observation that troglitazone inhibited phosphorylation of MEK1/2 suggests that troglitazone inhibits ERK1/2 activity by decreasing MEK1/2-dependent phosphorylation. The decrease in total MEK1/2 protein indicates that troglitazone

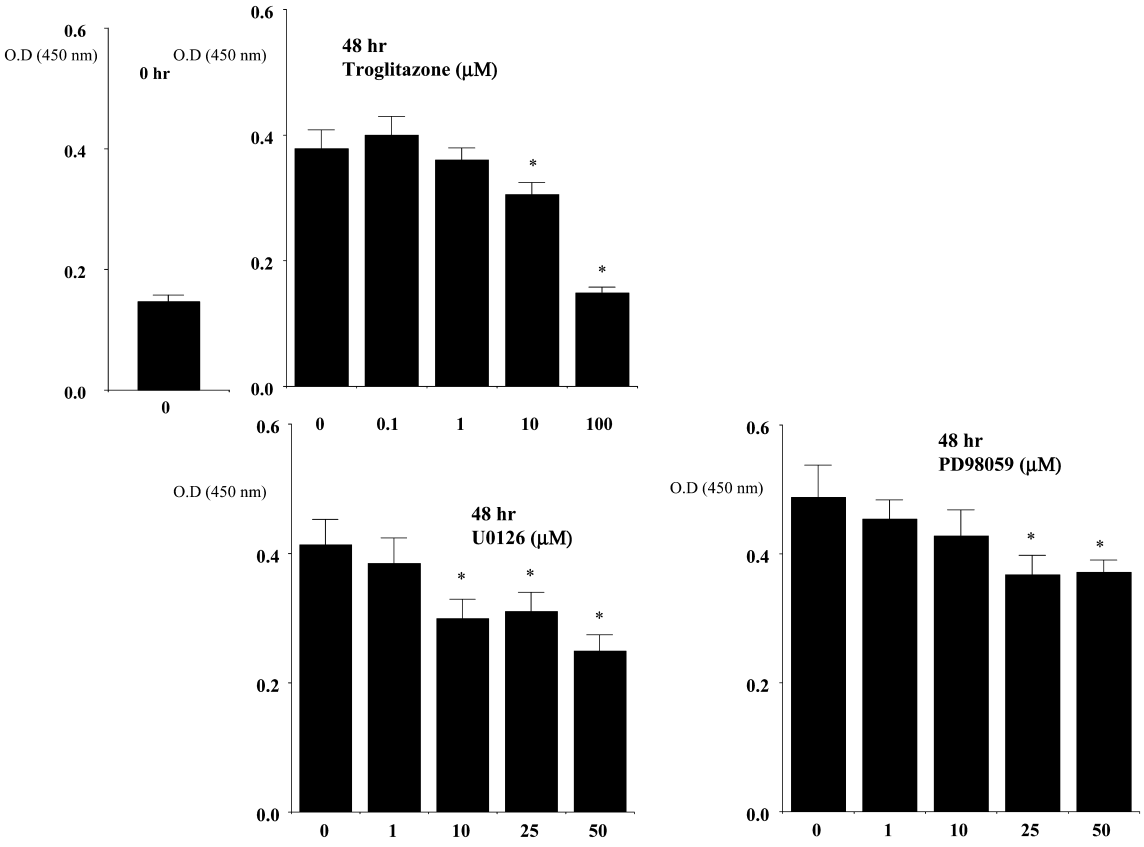

Fig. 4. Effect of troglitazone, U0126 or PD098059 on cell growth of human pancreatic cancer cells. PK-1 cells were treated with either troglitazone (100 μM), U0126 (50 μM) or PD098059 (50 μM) and cell numbers were determined by WST-1 assay at 0 or 48 h. Data are expressed as means ± SEM of five experiments. $*p < 0.01$ when compared with 0 (DMSO alone).

induces proteolytic cleavage or inhibits the transcription or translation of MEK1/2 protein, followed by suppression of MEK1/2 phosphorylation. These results suggest that the inhibition of ERK1/2 phosphorylation is mediated by down-regulation of MEK1/2 protein expression by troglitazone. In other words, the MEK–ERK signaling pathway is suppressed by troglitazone.

To further clarify whether inhibition of the MEK–ERK signaling is implicated in cell growth inhibition in human pancreatic cancer cells, we have performed a couple of experiments. Treatment with a MEK inhibitor, U0126 or PD98059, completely blocked ERK1/2 phosphorylation demonstrated in Fig. 3 and significantly inhibited cell growth in human pancreatic cancer cells, indicating that inhibition of the ERK1/2 signaling pathway by itself is sufficient to induce cell growth inhibition in PK-1 cells. Recent studies with other human pancreatic cancer cells have demonstrated that inhibition of ERK signaling by either U0126 or PD98059-induced growth arrest in MIA-Paca2 [33,34], being in good agreement with the present results. Based upon these results, we would suggest that inhibition of ERK1/2 signaling pathway mediates the cell growth inhibition by troglitazone in PK-1 cells.

With regard to the possible molecular mechanism by which PPARγ activation induces growth inhibition in human pancreatic cancer cells, we have already demonstrated that $p27^{Kip1}$, a CDKI, may be a key molecule that is implicated in the cell growth arrest by troglitazone in PK-1 cells because troglitazone increased the level of $p27^{Kip1}$ protein but not other CDKI, p21 or p18, protein, and the inhibition of cell proliferation by troglitazone was not observed in cells transfected with an antisense oligonucleotide against $p27^{Kip1}$ [16]. In addition, the present study provided an evidence that the MEK–ERK pathway is involved in the growth inhibition by troglitazone. One may therefore speculate that the MEK–ERK signaling pathway might mediate the increased $p27^{Kip1}$ protein expression that results in cell cycle arrest by troglitazone. It has been reported that inhibition of the MEK–ERK pathway by U0126 induced growth inhibition and increased $p27^{Kip1}$ protein level in human pancreatic cancer cells [33]. It was also shown that inhibition of the MEK–ERK pathway by PD98059 caused G1 arrest and increased $p27^{Kip1}$ expression in MIA-Paca2 [34]. These results suggest that there is a correlation between inhibition of the MEK–ERK activity and increased amount of $p27^{Kip1}$. There is an

evidence that ERK1/2 activity regulates $p27^{Kip1}$ level in melanoma cells [35], strongly indicating the functional relation between the MEK–ERK signaling and $p27^{Kip1}$ expression. These results suggest that inhibition of the MEK–ERK signaling pathway mediates the increased $p27^{Kip1}$ protein expression when cells are treated with troglitazone.

In conclusion, all these results suggest for the first time that down-regulation of MEK1/2–ERK1/2 signaling may be a mechanism of the cytotoxicity induced by troglitazone in human pancreatic cancer cells.

Acknowledgments

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