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Abstract

In the present study, we examined a role of mitogen-activated protein kinases (MAPKs), extracellular signal releted kinase (ERK), c-Jun N-terminal protein kinase (JNK) 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.

Key words: troglitazone, extracellular signal releted kinase,

mitogen-activated protein kinase kinase, pancreatic cancer cells,

growth inhibition, PPARγ

Introduction

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 in not only 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 play 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

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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 releted kinases (ERKs), c-Jun N-terminal protein linases (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, 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 PK-1 cells express PPARγ [1□]. 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 amphotericine, and 10 % fetal bovine serum.   Cells were incubated at 37℃ in a humidified atmosphere of 5 % CO_2 in air.

Chemicals

Troglitazone, a selective ligand for PPARγ, □□□□ was kindly provided from Sankyo Pharmaceutical Co. (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 were 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 µ g of protein were separated by 10% SDS-PAGE (PAG Mini DAIICHI, Daiichi Pure Chemicals, Tokyo, Japan). After electrophoresis, the proteins were transferred to PVDF membrane (Millipore Corporation, Bedford, MA, USA),

blocked overnight in Block Ace (Dainipponn Seiyaku, Osaka, Japan) at 4° C, reacted with primarily 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 hr, washed with TBS-T, reacted with secondary polyclonal antibody against rabbit IgG (Chemicon International, Temecula, CA, USA) for 1 hr 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 Internationa, 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

Inc., Corning, NY, USA) at a concentration of 2 x10^4/well in a volumes of 100 µl. Twenty four hour later, each well was incubated with troglitazone, U0126 or PD98059 at several concentrations for 0 or 48 hours. 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 mean ± SEM. Statistical analysis was performed by repeated measures ANOVA and subsequent Fisher's LSD test. P < 0.05 was considered statistically significant.

Results

Troglitazone inhibits ERK1/2 phosphorylation in human pancreatic cancer cells

Figure 1 shows that 24 hr after treatment of troglitazone, inhibition of ERK1/2 phosphorylation was observed while troglitazone failed to change 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 of 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 inhibit 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 Figure 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 hr to 48 hr (Figure 2B).

U0126 and PD98059 induces growth arrest in human pancreatic cancer cells

To clarify whether inhibition of ERK activity alone could inhibit cell growth in pancreatic cancer cells, effects of MEK inhibitors on cell growth was evalusted. Figure 3 demonstrated 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 μ M potently suppressed ERK phosphorylation at 24 and 48 hr. It was also shown that both MEK inhibitors, U0126 and PD98059 completely blocked ERK 1/2 phosphorylation throughout the period observed, indicating that the both chemicals block ERK activities. Using the inhibitors, WST-1 assay was

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performed to evaluate the effect of the chemicals on cell growth. As

illustrated in Figure 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 is

implicated in the cytotoxicity by troglitazone in human pancreatic and gastric

cancer cells [16, 17, 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. have demonstrated that U0126, the specific MEK inhibitor,

resulted in an inhibition of ERK phosphorylation and cytotoxicity in cultured

colon cancer cells [32]. Based on these evidence, 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 Figure 1 and Figure 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 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 superssed 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 perfomed a couple of experiments. Treatment with a MEK inhibitor, U0126 or PD98059 completely blocked ERK1/2 phosphorylation demonstrated in Figure 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 caner 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. Base upon these results, we would suggest that inhibition of ERK1/2 signalling 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.

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Figure legends

Figure 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 hr 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 μ

M troglitazone), lane 3 (1 μ M troglitazone), lane 4 (10 μ M troglitazone) or

lane 5 (100 μ M troglitazone), respectivrly.

Figure 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 hr 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 μ M troglitazone), lane 3 (1 μ M troglitazone), lane 4

(10 μ M troglitazone) or lane 5 (100 μ M troglitazone), respectivily. (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) hr after troglitazone

treatment.

Figure 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 hr (upper panel) and 48 hr (lower

panel). Each lane represents the result of DMSO control (Lane 1), 100 μ M

troglitazone (Lane 2), 50 μ M U0126 (Lane 3) or 50 μ M PD98059 (Lane 4),

respectively.

Figure 4

Effect of troglitazone, U0126 or PD098059 on cell growth of human

pancreatic cancer cells. PK-1 cells were treated with either troglitazone (100

μ M), U0126 (50 μ M) or PD098059 (50 μ M) and cell numbers were

determined by WST-1 assay at 0 or 48 hr. Data are expressed as the mean ±

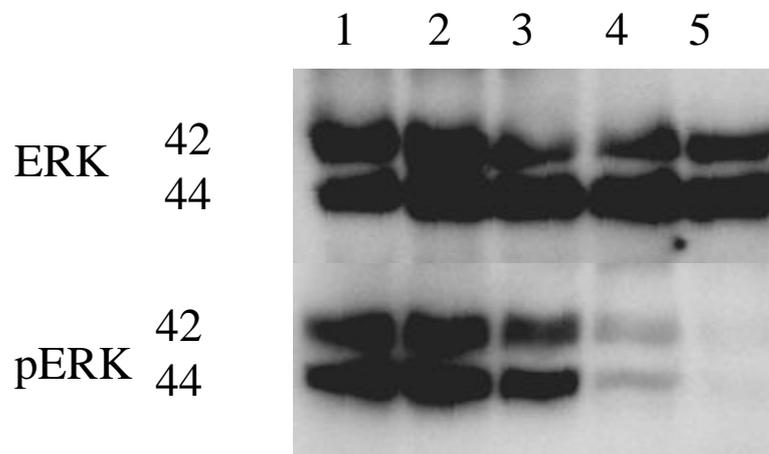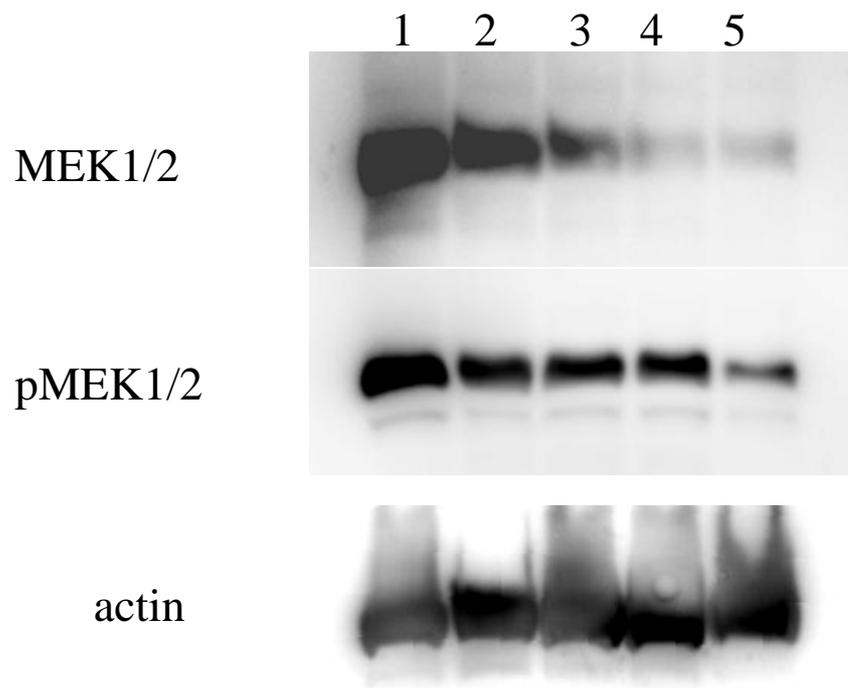SEM of five experiments. * $p < 0.01$ when compared with 0 (DMSO alone).
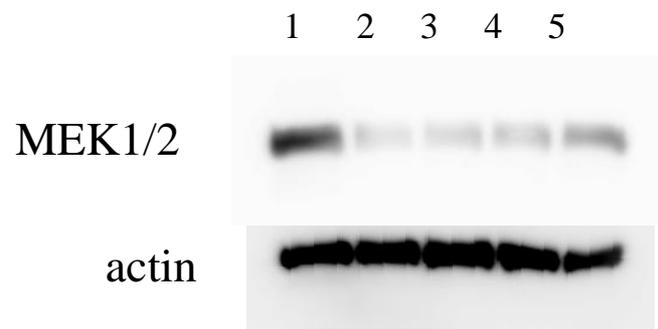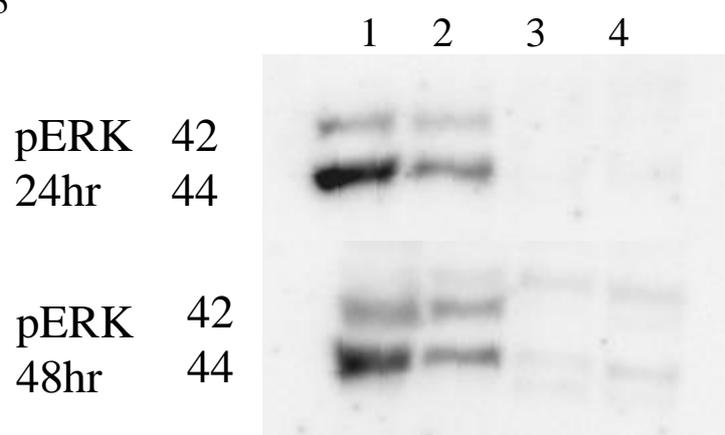
Figure 1

Figure 2

A

1 2 3 4 5

MEK1/2

pMEK1/2

actin

B

1 2 3 4 5

MEK1/2

actin

Figure 3

pERK 42
24hr 44

pERK 42
48hr 44

Figure 4