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Pyrrolidine dithiocarbamate-induced activation of ERK and increased expression of c-Fos in mouse embryonic stem cells

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Pyrrolidine dithiocarbamate (PDTC) is a stable anti-oxidant or pro-oxidant, depending on the situation, and it is widely used to inhibit the activation of NF-κB. We recently reported that PDTC activates the MIP-2 gene in a NF-κB-independent and c-Jun-dependent manner in macrophage cells. In this work, we found that PDTC activates signal transduction pathways in mouse ES cells. Among the three different mitogen-activated protein kinase (MAPK) pathways, including the extracellular-signal-regulated kinase (ERK), p38 MAP kinase, and stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) pathways, only the ERK pathway was significantly activated in mouse ES cells after stimulation with PDTC. Additionally, we observed a synergistic activation of ERK and induction of c-Fos after stimulation with PDTC in the presence of mouse embryonic fibroblast (MEF) conditioned medium. In contrast, another NF-κB inhibitor, BMS-345541, did not activate the MAP kinase pathways or induce expression of c-Fos. These results suggest that changes in the presence of the NF-κB inhibitor PDTC should be carefully considered when it used with mouse ES cells. [BMB reports 2009; 42(3): 148-153]

INTRODUCTION

Pyrrolidine dithiocarbamate (PDTC) (1) is a stable compound that acts as an antioxidant (2) or pro-oxidant (3) in different cell situations, and it is widely used to inhibit the activation of NF-κB. PDTC inhibits the expression of proinflammatory genes in response to inflammatory mediators such as TNF-α and LPS *in vivo* (4, 5) and *in vitro* (6, 7) by suppressing NF-κB activation. In contrast to these results, PDTC was reported to activate NF-κB (depending on its dose and the presence of metal ions) in PC12 cells (8). Furthermore, the plasma concentration of TNF-α was slightly augmented in PDTC-treated animals (9). Recently, we observed PDTC-induced expression of the proinflammatory cytokine gene MIP-2 in a mouse macrophage cell line, RAW 264.7, and investigated the involvement of the SAPK/JNK pathway and transcription factor AP-1 in this phenomenon (10).

Embryonic stem (ES) cells have the ability to proliferate extensively *in vitro* while maintaining their stemness on mouse embryonic fibroblast (MEF) cells (11, 12), and they also have demonstrated the capacity for pluripotent differentiation into multiple tissue lineages under certain conditions (13-15). Studies on ES cells are producing insights into embryonic development (16) and fate decision mechanisms including self-renewal, proliferation, and differentiation into specific lineages (17). Extrinsic changes in the environment surrounding ES cells and modulation of intrinsic properties including cellular signal transduction in ES cells may contribute to the fate decision of ES cells. Among several signal transduction pathways implicated in the self-renewal and differentiation of ES cells, the MAP kinase pathways ERK1/2, p38, and SAPK/JNK are known to regulate ES commitment from early stages of the process through mature differentiation (18).

NF-κB is a crucial molecule involved in diverse biological processes including embryo development, hematopoiesis, immune regulation, as well as neuronal functions via the induction of certain growth and transcription factors (19-22); however, little is known of NF-κB biology during ES cell functions. Previously, we reported that expression and activity of NF-κB is comparatively low in undifferentiated human ES cells, but its expression increases during differentiation of ES cells when induced by retinoic acid (23). Recently, we also confirmed this phenomenon in mouse ES cells (24), which suggest that low NF-κB expression and activity is relevant in different species, and that NF-κB may contribute to differentiation rather than self-renewal of ES cells. Accordingly, TNF-α (23, 24) and many other NF-κB agonists do not activate the NF-κB pathway in either human or mouse ES cells (our unpublished data). A recent report by other investigators also suggested a differentiation inducing activity of NF-κB in mouse ES cells (25). Therefore, we applied PDTC, an inhibitor of NF-κB signaling, during differentiation of mouse ES cells expecting inhibition of differentiation. However, we did not observe any clear protective effects on differentiation.

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As PDTC is known to activate many signaling pathways including MAP kinase pathways, which can influence on the fate decision of ES cells, we decided to check whether there is any unexpected effect of PDTC in ES cells. Here, we determined that PDTC, independent of NF-κB signaling, induced activation of the ERK1/2 pathway and c-Fos expression in mouse ES cells.

RESULTS AND DISCUSSION

Activation of MAP kinase ERK1/2 induced by PDTC

Although PDTC is a well-known inhibitor of the NF-κB signaling pathway at a broad range of concentrations (10 μM to 200 μM) depending on the cell type (4-8), it is also known to activate MAP kinase pathways in neuronal cells and macrophage cells. In this study, we assayed whether PDTC activates MAP kinase pathways in mouse ES cells by Western blot analysis using phospho-specific antibodies.

First, we placed mouse ES cells overnight in conditioned medium obtained from MEF feeder cells and then stimulated them with PDTC. Among the three different mitogen-activated protein kinase (MAPK) pathways tested, ERK1/2 was clearly activated in a time-dependent manner in the mouse ES cells following PDTC stimulation (Fig. 1A). Activation of p38 MAP kinase and SAPK/JNK was not observed (data not shown). When we stimulated the cells using different concentrations of PDTC, activation of ERK1/2 was observed to be dose-dependent (Fig. 1B). However, the viability of the cells was inhibited when stimulated at PDTC concentrations greater than 100 μM. Therefore, we used 50 μM of PDTC for all subsequent experiments.

ES cells are usually maintained on feeder cells that continuously supply growth factors including leukemia inhibitory factor (LIF). Therefore, PDTC's effect under actively growing conditions would be more physiologically significant. Here, we mimicked these condition by changing the medium with fresh conditioned medium in the presence or absence of PDTC and then checked for ERK activation. As shown in Fig. 2A and 2B, medium change alone induced a low level of ERK1/2 activation, and PDTC treatment in combination with media change induced a robust activation of ERK1/2. This indicates that PDTC can synergistically activate the ERK pathway along with other factors present in the conditioned medium. In order to see whether the effect of PDTC is general for all NF-κB inhibitors, we checked the effect of another NF-κB inhibitor, BMS-345541 [4(2′-aminoethyl)amino-1, 8-dimethylimidazo(1,2-a)quinoxaline], on mouse ES cells. BMS-345541 is a cell permeable and selective inhibitor of IKK-2 with an IC_{50} of about 300 nM (26). Previously we confirmed that in human ES-derived differentiated cells NF-κB activation induced by TNF-α was inhibited in the presence of 10–20 μM of BMS-345541 (23). Medium change with either a DMSO control or BMS-345541 did not induce an increased activation of ERK1/2 compared to the medium control (Fig. 2C and 2D). Furthermore, pretreatment with BMS-345541 did not influence

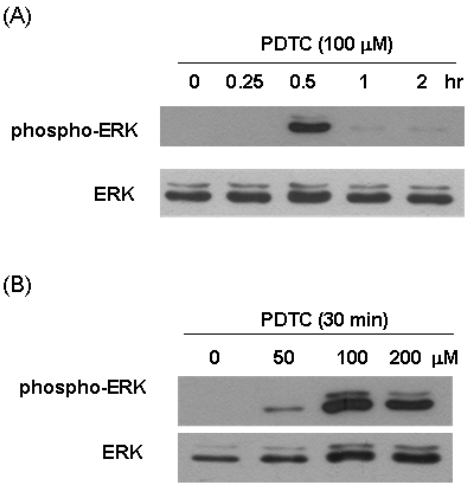

Fig. 1. Activation of ERK induced by PDTC alone in mouse ES cells. TC-1cells were stimulated with 100 μM of PDTC for the indicated periods (A) or stimulated for 30 min at the indicated concentrations of PDTC (B). Activation of MAP kinase pathways were examined by means of Western blotting using phospho-specific antibodies against ERK1/2, p38, or SAPK/JNK. The results of ERK1/2 analysis are shown. The amount of total ERK1/2 protein was shown as a loading control. This is a representative of three experiments with similar results.

PDTC-induced ERK activation (data not shown). Therefore, we can conclude that the PDTC activation of the ERK1/2 pathway is specific and is independent of the NF-κB signaling pathway in mouse ES cells.

We next examined whether MEF feeder cells are also activated by PDTC. The basal level of ERK activation was much higher in MEF cells and this is likely due to their production of autocrine factors including LIF. However, the increased ERK activation in response to PDTC was negligible. This implies that the effect of PDTC on feeder cells is limited and cell type-specific.

Induction of c-fos gene expression by PDTC in mouse ES cells

As activation of the ERK1/2 MAP kinase pathway induces the immediate early gene *c-fos* in many cells (27), we checked expression of *c-fos* after stimulation with PDTC in mouse ES cells. Here, we placed the cells overnight in MEF conditioned medium and stimulated them with PDTC directly after a fresh medium change. To investigate the mRNA expression of *c-fos* in PDTC-treated cells, RT-PCR assays were performed. As shown in Fig. 3, expression of the *c-fos* gene was barely detected in ES cells and was rapidly induced after PDTC stimulation. The expression level of *c-fos* mRNA was increased by PDTC, reaching its peak 2 hr after stimulation (Fig. 3A). Protein levels of *c-fos* were also increased by PDTC treatment (Fig. 3B). When we performed the same experiments with PDTC alone, without the medium change, we could not detect any *c-fos* induction. Therefore, it is likely that the effect of

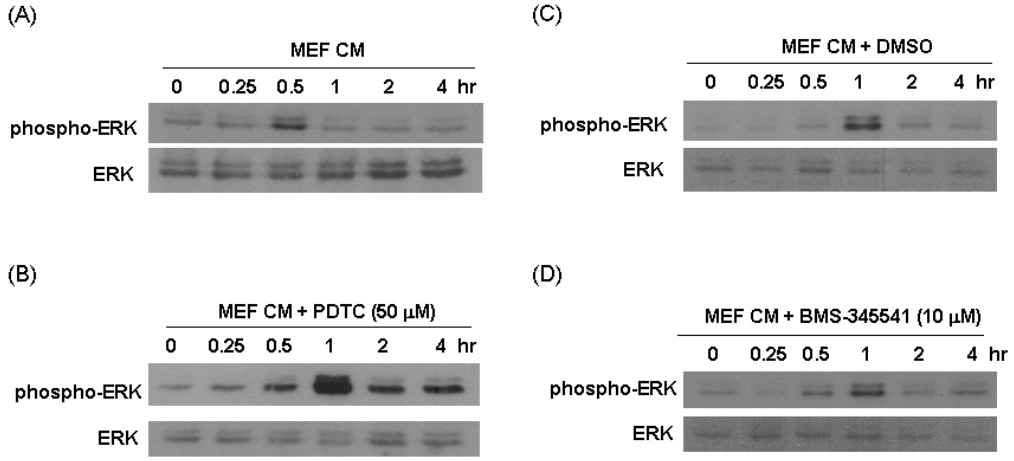

Fig. 2. Synergistic activation of ERK1/2 after stimulation with PDTC plus MEF conditioned medium in mouse ES cells. TC-1cells were stimulated with MEF conditioned medium in the presence of PBS (A), 50 μM of PDTC (B), DMSO (C), or 10 μM of BMS-345541 (D) for the indicated periods. Activation of ERK1/2 was examined by Western Blotting using phospho-specific antibodies against ERK1/2. The amount of total ERK1/2 protein was shown as a loading control. This is a representative of three experiments with similar results.

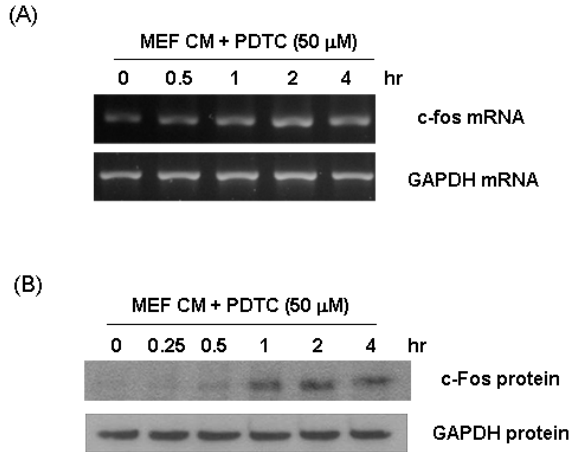

Fig. 3. Induction of c-Fos after stimulation with PDTC in mouse ES cells. TC-1 cells were stimulated with fresh conditioned medium plus PDTC for the indicated time periods, and expression of c-Fos was determined at the mRNA level as well as the protein level. (A) Levels of c-Fos mRNAs were examined by RT-PCR. (B) Expression of c-Fos protein was determined by Western blotting with an anti-c-Fos antibody. The amounts of GAPDH mRNA and protein were shown as a loading control. This figure is a representative of two experiments, both with similar results.

PDTC alone is weak, but it synergizes with the conditioned medium during induction of the *c-fos* protein. Medium change with either a DMSO control or BMS-345541 did not result in detectable induction of *c-fos*, which is in agreement with the lack of prominent ERK activation under these conditions (Fig. 2C and 2D).

Previously we have shown that PDTC treatment induced MIP-2 gene expression in the macrophage cell line RAW 264.7 (10). Here, MIP-2 gene expression was detected by PCR in mouse ES cells, but it was not induced by PDTC (data not shown). These results imply that gene regulation in response to PDTC is cell-type specific.

Embryonic stem cells can differentiate into multiple cell lineages *in vivo* and *in vitro*. The MAP kinase pathways ERK1/2, p38, and SAPK/JNK are known to regulate ES cell differentiation (18). In mouse ES cells, ERK1/2 inhibits the self-renewal and, accordingly, ES cells are protected from differentiation in the presence of the MEK1 inhibitor PD 98059 (28). However, upon commitment the ERK1/2 pathway is involved in development of extraembryonic tissues during early mesoderm differentiation and the formation of mature adipocytes. Recently, FGF stimulation of the ERK1/2 signaling cascade was implicated in the transition of mouse embryonic stem cells from self-renewal to lineage commitment (29). p38 is known to be involved in differentiation of several lineages including cardiomyocyes, myotubes, and adipocytes. The JNK1 pathway has been implicated in ectodermal and enodermal differentiation (18). The results presented in this study reveal that PDTC can activate ERK1/2 in mouse ES cells, and this activation is synergistically enhanced in the presence of conditioned medium. Therefore, PDTC might affect self-renewal or differentiation by modulating the balance of signals influencing mouse ES cells.

Mouse and human ES cells have similar features. They express classical markers of pluripotent stem cell lines such as Oct4, Nanog, alkaline phosphatase (AP), and high levels of telomerase activity (11, 12). However, they are different in morphology, patterns of embryonic antigen immunostaining, expression of differentiation markers, and population doubling times (30). Mouse ES cells can be cultured and kept in an undifferentiated state and maintain their pluripotency with cul-

ture medium containing LIF. In contrast, bFGF and ERK activation was implicated in the maintenance of human ES cells (31, 32). Therefore, the results from mouse ES cells may have limited translation to human research. To check whether the effect of PDTC is general among ES cells form different species, we checked its effect on human ES cells under the same conditions as with mouse ES cells. However, we did not observe any significant activation of ERK1/2 in human ES cells (data not shown). Therefore, PDTC's effect may be different in mouse and human ES cells.

NF-κB is an important transcription factor involved in diverse biological activities (33). The contribution of NF-κB in self-renewal of ES cells is unconvincing. Recently, it was reported that NF-κB, a differentiation-inducing factor, is suppressed by the essential stem cell regulator Nanog in mouse ES cells (25). Our own results from human and mouse ES cells are in accordance with this observation; NF-κB expression and activity are lower in ES cells and are increased during differentiation induced by retinoic acid (23, 24). We can use chemical inhibitors for studying the possible impact of a particular signal transduction pathway; however, the inhibitor itself can activate the cells and affect the outcome independently of the known signal transduction pathway on which it acts (34). In this study, we have presented activation of signal transduction and induction of gene expression by PDTC in mouse ES cells. Considering the results shown here, use of PDTC to assay the possible contribution of NF-κB in self-renewal or differentiation during cell culture may have potential problems in that PDTC-induced ERK1/2 activation may contribute to differentiation. In conclusion, our results suggest that consequences of PDTC treatment should be carefully considered, at least in mouse ES cells.

MATERIALS AND METHODS

Maintenance of mouse ES cells

The mouse ES cell line TC-1 was maintained on mouse embryonic fibroblasts (MEF) in ES medium, which contained Dulbecco's modified Eagle's medium (DMEM), 15% fetal bovine serum (FBS; Hyclone Inc., Logan, UT, USA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cultures were passaged as cells became confluent (i.e., about two or three times a week). ES cells were dissociated with 0.025% trypsin-EDTA and then seeded on MEF prepared as follows: MEF cells were harvested and irradiated with 30 Gy and then seeded at a density of approximately 5.5 x 10^4 cells/ml in MEF medium (DMEM, 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids) one day before ES cell seeding.

Stimulation of mouse ES cells with PDTC

In order to examine intracellular signaling in mouse ES cells in response to PDTC, cells were grown overnight on a gelatin-coated plate in the presence of MEF conditioned medium. To check the effect of PDTC alone, cells were directly treated with PDTC at the indicated concentration. To check the effect of PDTC in synergy with MEF conditioned medium, culture medium was changed with fresh conditioned medium and the cells were treated with PDTC at a concentration of 50 μM. When necessary, mouse ES cells were treated with the IKK inhibitor BMS-345541 at a concentration of 10 μM.

RT-PCR analysis

Total RNA was isolated using TRI REAGENT® according to the instructions provided by the manufacturer (MRC, Cincinnati, OH, USA). 5 μg of total RNA was reverse-transcribed in first-strand buffer containing 6 μg/ml oligo(dT) primer, 50 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 2 mM dNTP, and 40 U RNase block ribonuclease inhibitor. The reaction was conducted at 37°C for 50 min. One microliter of the cDNA synthesis reaction was subjected to standard PCR for 30 or 35 cycles of denaturation for 60 sec at 95°C, annealing for 60 sec at 58ºC, and elongation for 60 sec at 72°C. The primer sequences are as followings: Mouse c-fos, 5′-GAATAAGATGGCTGCAGCCAAGTGCCGGAA-3′ and 5′-CAGTCAAATCCAGGGAGGCCACAGACATCT-3′ (236 bp product); mouse MIP-2, 5′-TGGGTGGGATGTAGCTAGTTCC-3′ (sense) and 5′-AGTTTGCCTTGACCCTGAAGCC-3′ (anti-sense) (466 bp product); and mouse GAPDH, 5′-primer 5′-ACCACAGTCCATGCCATCAC-3′ and 3′-primer 5′-TCCACCACCCTGTTGCTGTA-3′ (477 bp product).

Western blotting

Cells on cell culture plates were washed with PBS once and harvested using cell scrapers. After centrifugation, cells were resuspended in 50 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/mL aprotonin, 10 mM EDTA, 10 μg/mL leupeptin, 100 mM NaF, 2 mM Na_3VO_4, and 1% NP-40). Equal amounts of protein were loaded on sodium dodecyl sulfate polyacrylamide gel, subjected to electrophoresis (SDS-PAGE), and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, Bedford, MA, USA). Membranes were blocked with 5% dry milk and probed with the appropriate primary antibody. Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and an ECL reagent (iNtRon, Seongnam, Korea). Membranes were stripped and then probed with another primary antibody when necessary. Antibodies to ERK1/2, phospho-ERK1/2 (Thr202/ Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p38, and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to c-Fos and GAPDH were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

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