The role of dual-specificity phosphatase 1 and protein phosphatase 1 in β_2 -adrenergic receptor-mediated inhibition of extracellular signal regulated kinase 1/2 in triple negative breast cancer cell lines

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Abstract. Triple negative breast cancer cell lines express high levels of β_2 -adrenergic receptor, which have a significant influence on the activity of extracellular signal-regulated kinase (ERK)1/2. Therefore, it is important to understand the link between β_2 -adrenergic receptor signaling and ERK1/2 activity in terms of cancer cell regulation and cancer progression. Although the molecular mechanisms are not completely clarified, β_2 -adrenergic receptor stimulation appears to reduce the basal levels of phosphorylated (p)ERK1/2 in MDA-MB-231 breast cancer cells. The aim of the current study was to determine the mechanism of β_2 -adrenergic receptor-mediated ERK1/2 dephosphorylation by investigating the role of dual-specificity phosphatase (DUSP)1/6 and protein phosphatase (PP)1/2, which are established regulators of ERK1/2 phosphorylation, in MDA-MB-231 and MDA-MB-468 breast cancer cell lines. (E)-2-benzylidene-3-(cyclohexylamino)-2,3dihydro-1H-inden-1-one (BCI) and calyculin A were employed as DUSP1/6 and PP1/PP2 inhibitors, respectively. Subsequently, the protein levels of DUSP1, PP1, pPP1, ERK1/2 and pERK1/2 were measured by western blot analysis. Cells

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Abbreviations: BCI, 2-benzylidene-3-(cyclohexylamino)-2,3dihydro-1H-inden-1-one; DUSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PP, protein phosphatase

Key words: β_2 -adrenergic receptor, dual-specificity phosphatase 1, serine/threonine phosphatases, protein phosphatase 1, extracellular signal-regulated kinase 1/2, calyculin A, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one, MDA-MB-231, MDA-MB-468, triple negative breast cancer cells

were transfected with DUSP1 small interfering (si)RNA or PP1 siRNA to inhibit their expression. The results demonstrated that β_2 -adrenergic receptor agonists led to the dephosphorylation of basal pERK1/2 in MDA-MB-231 and MDA-MB-468 cells. The DUSP1/6 inhibitor, BCI, and the PP1/PP2 inhibitor, calyculin A, antagonized the β_2 -adrenergic receptor-mediated dephosphorylation of ERK1/2. Furthermore, β_2 -adrenergic receptor stimulation increased the protein expression level of DUSP1, with no effects on DUSP6, PP1 and PP2 expression, and enhanced the expression of the active form of PP1. Downregulation of the expression of DUSP1 or PP1 led to a decline in the \u03b32-adrenergic receptor-mediated dephosphorylation of ERK1/2. The results of the present study indicate that β_2 -adrenergic receptor-mediated dephosphorylation of ERK1/2 may be associated with the activity of DUSP1 and PP1 in MDA-MB-231 and MDA-MB-468 triple negative breast cancer cell lines. The clinical importance of β_2 -adrenergic receptor-mediated inactivation of ERK1/2 as well as the activation of DUSP1 and PP1 should be carefully evaluated in future studies, particularly when β_2 -adrenergic blockers are used in patients with triple negative breast cancer.

Introduction

The sympathetic nervous system, catecholamines and adrenergic receptors function in cellular growth, differentiation and the regulation of tumor growth and cancer progression (1-6). Although the expression of β -adrenergic receptors in breast cancer cells has been well-documented and extensively investigated (7-13), the regulatory roles of these receptors in breast cancer cell replication and proliferation are not fully understood at present.

One of the key regulators of cellular growth and differentiation, and tumor progression and invasion, is extracellular signal-regulated kinase (ERK)1/2. β_2 -adrenergic receptor stimulation regulates the activity of ERK1/2 and, depending on the cell type, activates ERK1/2 (14,15). On the other hand, in certain cancer cell lines, including MDA-MB-231 breast cancer cells, β_2 -adrenergic receptor stimulation inhibits ERK1/2 phosphorylation, causing its inactivation (16,17). There are two key processes that determine the phosphorylation and activity status of ERK1/2: Its phosphorylation rate by kinases and its dephosphorylation rate by phosphatases (18). Inhibition of the activity of kinases, which phosphorylate ERK1/2, and/or activation of phosphatases, which dephosphorylate pERK1/2, may be induced by β_2 -adrenergic receptor stimulation, which will reduce phosphorylated (p)ERK1/2 levels in MDA-MB-231 cancer cell lines.

Carie and Sebti (16) reported that inhibition of ERK1/2 phosphorylation induced by β_2 -adrenergic receptor stimulation is mediated by inactivation of Raf-1 proto-oncogene/ mitogen-activated protein kinase (MAPK)1 kinases by a cyclic AMP-dependent pathway in MDA-MB-231 cells. This indicates that inhibition of kinases is implicated in β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. However, certain reports have indicated that β -adrenergic receptor stimulation may also influence the activity of various phosphatases. *β*-adrenergic receptor stimulation increases the expression of mitogen-activated dual-specificity phosphatase (DUSP/MKP)1, which may mediate the rapid dephosphorylation of ERK1/2 in the rat pineal gland (19). Another study that investigated the phosphorylation status of several signaling proteins in mouse embryonic fibroblast cells demonstrated that β-adrenergic receptor stimulation causes the dephosphorylation of protein phosphatase (PP)1 at tyrosine 320, leading to its activation (20). However, to the best of our knowledge, no associations between the activity of these phosphatases and β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation in cancer cell lines have been previously reported. Considering the important roles of β_2 -adrenergic receptor signaling and the activity status of phosphatases in the regulation of cancer cell lines, we hypothesize that determining the association between the β_2 -adrenergic receptor and these phosphatases may contribute to an improved understanding of breast cancer cell regulation.

Therefore, the present study focused on investigating the role of phosphatases in β_2 -adrenergic receptor-mediated dephosphorylation of ERK1/2 in breast cancer cells. MDA-MB-231 and MDA-MB-468 triple negative breast cancer cell lines, which are negative for the estrogen receptor (ER-), progesterone receptor (PR-) and human epidermal growth factor receptor 2 (HER2-), were employed in the present study as these cells express high levels of the β_2 -adrenergic receptor (12) and exhibit a high activity of ERK1/2, as high levels of pERK1/2 are present (21). In addition, as other breast cancer cell lines differ in terms of the expression of HER2, ER and PR and may lead to variability. Therefore, to reduce variability, the present study performed the experiments in two triple negative breast cancer cell lines. One important group of pERK1/2 phosphatases is DUSPs. There are nine different DUSPs, of which DUSP6/MKP3 is primarily cytosolic. DUSP6 is able to interact with ERK1/2 and cause dephosphorylation, which ultimately inactivates it (22,23). DUSP1 is a phosphatase that is localized in the nucleus and is also involved in the regulation of ERK1/2 activity (22,24). The other protein serine/threonine phosphatases, PP1 and PP2, also have regulatory roles in the dephosphorylation of ERK1/2 (25). The present study investigated the roles of DUSP1/6 and PP1/2 in β_2 -adrenergic receptor-mediated ERK1/2 dephosphorylation in MDA-MB-231 and MDA-MB-468 cells by using a DUSP1/6 inhibitor, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI), and a PP1/2 inhibitor, calyculin A, and by determining the expression level of these phosphatases and reducing their expression level by transfection of small interfering (si)RNA.

Materials and methods

Materials. The β_2 ligands terbutaline, clenbuterol, formoterol, epinephrine, isoproterenol and ICI118,551 hydrochloride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were dissolved in saline. The DUSP1/6 inhibitor, BCI (Merck KGaA), and the PP1/2 inhibitor, calyculin A (Cell Signaling Technology, Inc., Danvers, MA, USA), were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and diluted in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Capricorn Scientific, Ebsdorfergrund, Germany). The final concentration of DMSO did not exceed 0.2%. In all control experiments, cells were incubated with the corresponding dilution of solvent used for the ligand. The antibodies used were as follows: Total (t)PP1 catalytic subunit α (PP1A; 1:1,000; Sc-271762; Santa Cruz Biotechnology, Inc., TX, USA), tPP2 (PP2A; 1:1,000; ab33537; Abcam, Cambridge, UK), pPP1A (T320; 1:1,000; ab6234; Abcam), pPP2A (Y307; 1:1,000; Sc-12615, Santa Cruz Biotechnology, Inc.), pERK1/2 (1:5,000; Sc-16982; Santa Cruz Biotechnology, Inc.), tERK1/2 (1:5,000; Sc-154; Santa Cruz Biotechnology, Inc.), MKP1/DUSP1 (1:1,000; Sc-1102; Santa Cruz Biotechnology, Inc.), MKP3/DUSP6 (1:1,000; Sc-377070; Santa Cruz Biotechnology, Inc.), GAPDH (1:5,000; Sc-166545; Santa Cruz Biotechnology, Inc.), goat anti-rabbit (1:10,000; Sc-31460; Santa Cruz Biotechnology, Inc.), rabbit anti-goat (1:10,000; Sc-2768; Santa Cruz Biotechnology, Inc.) and donkey anti-mouse (1:10,000; Sc-2314; Santa Cruz Biotechnology, Inc.).

Cell culture and stimulation. The cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 and MDA-MB-468 cells were cultured in 75 cm² non-treated cell culture flasks in DMEM/F12 (Capricorn Scientific) enriched with 10% fetal bovine serum (Capricorn Scientific) and 1% penicillin (10,000 I.U/ml; Biochrom GmbH, Berlin, Germany) and 1% streptomycin $(10,000 \,\mu\text{g/ml};\text{Biochrom GmbH})$ at 5% CO₂, 37°C with 90-95% humidity. For each experiment, cells (2.5x10⁵ cells/well) were plated in a 6-well plate at 37°C with treatments performed on the second day following overnight serum starvation. The following treatments were included in the present study: Terbutaline, 1 μ M for 2, 5, 10 or 30 min; clenbuterol, 1 μ M for 10 min; formoterol, 0.1 μ M for 10 min; isoproterenol, 1 μ M for 2, 5, 10 or 30 min; and epinephrine, 10 μ M for 10 min. In certain experiments, prior to β_2 adrenergic stimulation, cells were pretreated for 30 min at 37°C with one of the following inhibitors: ICI118,551 hydrochloride (0.1 μ M), BCI (10 μ M) and calyculin A (10 nM).

RNA interference. DUSP1 siRNA (cat. no. Sc-35937), PP1 siRNA (cat. no. Sc-36299), negative control siRNA (cat. no. Sc-3707), transfection reagent (cat. no. Sc-29528) and

medium (cat. no. Sc-36868) were all purchased from Santa Cruz Biotechnology, Inc. The cells (2.5×10^5) were cultured in a 6-well-plate and 24 h later, when the cells reached 70% confluency, DUSP1 siRNA (1 μ M), PP1 siRNA (1 μ M) or negative control siRNA (1 μ M) were transfected according to the manufacturer's protocol. After transfection for 24 h at 37°C, the medium was replaced and the cells were cultured for an additional 24 h. The transfected cells were stimulated with 0.1 or 1 mM (data not shown) terbutaline (5-10 min) or saline. pERK1/2, tERK, DUSP1, PP1 and GAPDH levels were subsequently determined by western blot analysis.

Protein isolation and western blotting. Following treatments, cells were immediately placed on ice, washed with ice-cold PBS and homogenized in 100 µl lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany) containing 1% Nonidet P40, 0.02 M sodium orthovanadate and protease inhibitors. Following homogenization, cells were incubated for 15 min and centrifuged at 5,000 x g for 5 min at 4°C. The supernatant was collected, protein concentration was determined using the Bradford protein assay and stored at -80°C. Electrophoresis (20-30 μ g protein/per lane) was performed on newly-cast 8-10% sodium dodecyl sulphate (SDS)-polyacrylamide gels followed by transfer onto polyvinylidene difluoride membranes. The membranes were blocked for 2 h at 22°C in PBS with 20 mM NaH₂PO₄-Na₂HPO₄ (pH 7.6) containing 154 mM NaCl, 5% nonfat dry milk and 0.1% Tween-20. The membranes were incubated with the appropriate primary antibodies overnight at 4°C and washed three times for 10 min with TBS-0.2% Tween-20 prior to incubation for 1 h at 22°C with horseradish peroxidase conjugated anti-rabbit, anti-mouse or anti-goat secondary antibody. Following washing, the membranes were soaked in Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and imaged using a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.). Band intensities were quantified using Image Lab software (version 5; Bio-Rad Laboratories, Inc.). Two separate bands were observed for pERK1/2 (42 and 44 kDa) and the sum of the intensities of these two bands were determined. When required, membranes were stripped with 2% SDS and 0.7% mercaptoethanol in 10 ml PBS prior to incubation with another antibody overnight. Band intensities were presented relative to tERK or GAPDH expression.

Statistical analysis. Data are presented as the mean \pm standard error of the mean. n represents the number of independent experiments for each indicated condition. Statistical analysis was performed using SPSS 17.0 for Windows software (SPSS, Inc., Chicago, IL, USA). Statistical analysis of data and comparisons between multiple groups were performed by one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

 β_2 -adrenergic receptor stimulation and pERK1/2 dephosphorylation in MDA-MB-231 and MDA-MB-468 cells. As expected, two separate bands for pERK1/2 (42 and 44 kDa) were generally observed. However, for certain measurements, due to the high level of pERK1/2 in MDA-MB-231 or MDA-MB-468, one large, combined band was obtained located in the range of 42-44 kDa.

The results in Fig. 1 confirmed that β_2 -adrenergic receptor stimulation led to the dephosphorylation of pERK1/2. β_2 -adrenergic receptor-selective agonists, terbutaline (1 μ M), formoterol (0.1 μ M) and clenbuterol (1 μ M), and nonselective β -adrenergic receptor agonists, epinephrine (10 μ M) and isoproterenol (1 μ M), all dephosphorylated pERK1/2 in MDA-MB-231 (Fig. 1A) and MDA-MB-468 (Fig. 1B) cells. Furthermore, pERK1/2 dephosphorylation induced by terbutaline was blocked by the β_2 -adrenergic receptor antagonist ICI118,551 hydrochloride (0.1 μ M), indicating that this response is primarily mediated by the β_2 -adrenergic receptor (Fig. 1). pERK1/2 dephosphorylation was observed following treatment with 10 nM-1 μ M terbutaline (data not shown).

The DUSP1 inhibitor, BCI, antagonizes β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. The activation of DUSP1/6 is reported to lead to the dephosphorylation of pERK1/2 and negatively regulate its activity (22-24). MDA-MB-231 and MDA-MB-468 cells were treated with the DUSP1/6 inhibitor, BCI (10 μ M), for 30 min prior to terbutaline (1 μ M) stimulation. BCI treatment of the cells completely antagonized β_2 -adrenergic receptor-mediated inhibition of ERK1/2 phosphorylation in MDA-MB-231 (Fig. 2A) and MDA-MB-468 (Fig. 2B) cells.

 β_2 -adrenergic receptor stimulation induces the expression of DUSP1. As DUSP1 is an inducible protein (19,22,26,27), the present study also investigated DUSP1 expression levels by western blot analysis with or without terbutaline (1 μ M) or isoproterenol (1 μ M) treatment. Terbutaline and isoproterenol treatment (5, 10 and 30 min) increased the protein expression of DUSP1 in MDA-MB-231 cells, compared with control treatment (Fig. 3A). In addition, DUSP1 protein expression was also increased in MDA-MB-468 cells following 10 min terbutaline treatment (Fig. 3B). However, the results in Fig. 4 indicate that DUSP6 levels in MDA-MB-231 cells were not altered by terbutaline treatment for 2-30 min.

Downregulation of DUSP1 reduces β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. Western blotting results in Fig. 5 indicate that DUSP1 siRNA transfection successfully reduced the expression of DUSP1 compared with the control siRNA transfection group in MDA-MB-231 and MDA-MB-468 cells. Terbutaline-mediated dephosphorylation of pERK1/2 significantly declined following downregulation of DUSP1, compared with the control siRNA transfection group. While 0.1 μ M terbutaline led to 75±3 and 70±5% dephosphorylation of ERK1/2 in MDA-MB-231 and MDA-MB-468 cells, respectively, dephosphorylation was 43 ± 7.9 and $47\pm6\%$, respectively, following downregulation of DUSP1 (Fig. 5). In the present study, 0.1 and 1 μ M terbutaline were generally employed. There was no significant difference in the 0.1 or 1 μ M terbutaline-induced pERK1/2 dephosphorylation (data not shown). Terbutaline treatment following downregulation of DUSP1 was performed with 0.1 and 1 (data not shown) μ M terbutaline, and results demonstrated that the inhibition of dephosphorylation of pERK1/2 was pronounced



Figure 1. β_2 -adrenergic receptor agonists led to the dephosphorylation of pERK1/2 in MDA-MB-231 and MDA-MB-468 cells. Treatment with the β_2 -adrenergic receptor-selective agonists terbutaline (1 μ M), clenbuterol (1 μ M) and formoterol (0.1 μ M), and nonselective β -adrenergic receptor agonists isoproterenol (1 μ M) and epinephrine (10 μ M), for 10 min significantly inhibited the basal level of pERK1/2 in (A) MDA-MB-231 and (B) MDA-MB468 cells. Pretreatment of cells with the β_2 -adrenergic receptor antagonist, ICI118,551 hydrochloride (0.1 μ M) for 30 min, completely antagonized terbutaline-stimulated pERK1/2 dephosphorylation in both cell lines. Representative western blot bands for pERK and tERK are presented. Two replicate bands are presented for certain treatment groups. tERK bands were used as a reference, and pERK band intensities were normalized to tERK and presented as a percentage of saline-treated control cells. Data are presented as the mean \pm standard error of the mean, n=4-5. *P<0.05 vs. control, #P<0.05 vs. Ter group. ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; tERK, total ERK; C, control; Ter, terbutaline; Fmt, formoterol; Clen, clenbuterol; Epi, epinephrine; Iso, isoproterenol; ICI, ICI118,551 hydrochloride.



Figure 2. The DUSP1 inhibitor, BCI, antagonized β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. Levels of pERK following treatment with the β_2 -adrenergic receptor-selective agonist terbutaline (1 μ M) for 10 min with or without pretreatment with the DUSP1/6 inhibitor BCI (10 μ M) for 30 min in (A) MDA-MB-231 and (B) MDA-MB-468 cells. BCI antagonized β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation in both cell lines. Representative western blot bands for pERK and tERK are presented. Two replicate bands are presented for certain treatment groups. pERK band intensities were normalized to tERK and presented as a percentage of saline + 0.1% DMSO-treated control cells. DMSO (0.1%) was the final dilution of the solvent of BCI and was used as the control for pretreatment with BCI. Data are presented as the mean ± standard error of the mean, n=4-5. *P<0.05 vs. control, *P<0.05 vs. Ter. DUSP, dual-specificity phosphatase; BCI, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; tERK, total ERK; DMSO, dimethyl sulfoxide; C, control; Ter, terbutaline.



Figure 3. β_2 -adrenergic receptor stimulation induced the expression of DUSP1. (A) The protein expression of pERK, tERK, DUSP1 and GAPDH was investigated following treatment with terbutaline (1 μ M) or isoproterenol (1 μ M) treatment for 2-30 min in MDA-MB-231 cells. (B) The expression of pERK, tERK, DUSP1 and GAPDH following treatment with terbutaline (1 μ M) for 10 min in MDA-MB-468 cells. Representative western blot bands for pERK, tERK, DUSP1 and GAPDH are presented. In both cell lines, β_2 -adrenergic receptor stimulation increased the expression level of DUSP1. Band intensities were normalized to GAPDH for DUSP1 and tERK for pERK, and presented as a percentage of saline-treated control cells. Data are presented as the mean \pm standard error of the mean, n=4-5. *P<0.05 vs. control cells. DUSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; tERK, total ERK; C, control; Ter, terbutaline; Iso, isoproterenol.



Figure 4. β_2 -adrenergic receptor stimulation did not induce the expression of DUSP6. The expression of DUSP6 protein in MDA-MB-231 cells following treatment with terbutaline is presented. MDA-MB-231 cells were treated with terbutaline (1 μ M) for 2-30 min and the expression of DUSP6 and GAPDH was determined by western blotting. Representative western blot bands for DUSP6 and GAPDH are presented. Band intensities were normalized to GAPDH for DUSP6 and presented as a percentage of saline-treated control cells. Data are presented as the mean \pm standard error of the mean, n=4. DUSP, dual-specificity phosphatase; C, control.

when 0.1 μ M terbutaline was employed. Therefore, results for 0.1 μ M terbutaline treatment are presented in Fig. 5. We hypothesized that signal strength is lower when a lower concentration of terbutaline (0.1 μ M) was employed and, therefore, a lower concentration of terbutaline-mediated ERK1/2 dephosphorylation was more sensitive to the down-regulation of DUSP1 levels. Therefore, terbutaline-mediated pERK1/2 dephosphorylation may depend on the expression level of DUSP1.

The PP1 inhibitor, calyculin A, antagonizes β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. Additional phosphatases that regulate ERK1/2 phosphorylation are the serine/threonine phosphatases PP1 and PP2. Therefore, the present study also investigated the potential involvement of PP1 and PP2 in β_2 -adrenergic receptor-mediated dephosphorylation of pERK1/2. To investigate their roles, calyculin A, a PP1/2 inhibitor, was employed. The results demonstrated that terbutaline-mediated ERK1/2 dephosphorylation was reversed by 30 min pretreatment of MDA-MB-231 and MDA-MB-468 cells with 10 nM calyculin A (Fig. 6).

 β_2 -adrenergic receptor stimulation enhances the expression of the active form of PP1. Phosphorylation of PP1 at tyrosine 320 and PP2 at tyrosine 307 represent inhibited forms of these phosphatases, whereas dephosphorylation of PP1 (tyrosine 320) and PP2 (tyrosine 307) are associated with enhanced activity of these phosphatases (28,29). The present study investigated the expression levels of the phosphorylated forms of PP1 and PP2 following stimulation with terbutaline (1 μ M). The results demonstrated that the phosphorylation of PP1 was inhibited following 2-10 min treatment with terbutaline in MDA-MB-231 cells (Fig. 7). Furthermore, a decrease in the level of pPP1 was observed following terbutaline



Figure 5. Downregulation of DUSP1 reduced β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. Results demonstrating the expression levels of DUSP1 following transfection with control (1 μ M) or DUSP1 (1 μ M) siRNA, and pERK expression in control and DUSP1 siRNA-transfected cells following treatment with terbutaline (0.1 μ M) or saline, are presented for (A) MDA-MB-231 and (B) MDA-MB-468 cells. Representative western blot bands for pERK, tERK, DUSP1 and GAPDH are presented. Band intensities were normalized to GAPDH for DUSP1 and tERK for pERK, and presented as a percentage of saline-treated control cells. Bar graphs indicate the percentage of dephosphorylated ERK1/2 induced by terbutaline treatment in control or DUSP1 siRNA-transfected cells. Data are presented as the mean ± standard error of the mean, n=4. *P<0.05 vs. control cells. DUSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; siRNA, small interfering RNA; tERK, total ERK; csiRNA, control siRNA; siDUSP1, siRNA targeting DUSP1; C, control; Ter, terbutaline.

treatment, while pPP2 levels were not significantly altered, in MDA-MB-231 cells (Fig. 7). However, terbutaline did not alter the expression levels of tPP1 and tPP2 in MDA-MB-231 cells (Fig. 7). In MDA-MB-468 cells, 1 μ M terbutaline for 10 min also reduced pPP1 levels without affecting tPP1 levels (Fig. 8). These results indicate that by enhancing the expression of the active form of PP1, terbutaline stimulation may contribute to ERK1/2 dephosphorylation.

Downregulation of PP1 reduces β_2 -adrenergic receptormediated pERK1/2 dephosphorylation. The present study also downregulated PP1 expression by transfecting cells with PP1 siRNA. Western blotting results in Fig. 9 demonstrated that PP1 was successfully downregulated following transfection with PP1 siRNA, compared with the control siRNA transfection group, in MDA-MB-231 and MDA-MB-468 cells. Terbutaline (0.1 μ M)-mediated pERK1/2 dephosphorylation was significantly reduced following downregulation of PP1 expression, compared with the control siRNA transfection group (Fig. 9). Similar to the results observed in the DUSP1 downregulation experiments, while 0.1 μ M terbutaline caused 76±4 and 70±5% dephosphorylation of ERK1/2 in MDA-MB-231 and MDA-MB-468 cells, respectively, these values reduced to 44±6 and 30±10%, respectively, following downregulation of PP1. Experiments involving the down-regulation of PP1 employed 0.1 and 1 μ M (data not shown) terbutaline and, similar to the results for the downregulation of DUSP1, a lower concentration of terbutaline (0.1 μ M) was selected. ERK1/2 dephosphorylation was more sensitive to the downregulation of PP1 levels at this lower concentration. Therefore, terbutaline-mediated pERK1/2 dephosphorylation may depend on the expression levels of both PP1 and DUSP1.

Discussion

MDA-MB-231 and MDA-MB-468 triple negative breast cancer cell lines express the β_2 -adrenergic receptor, and the results of several previous studies indicate that the β_2 -adrenergic receptor signaling pathway may be involved in tumor development, metastasis and cancer progression (4,5,13). DUSP1 and PP1 are also reported to be involved in the regulation of cell proliferation, and cancer development and progression, and they have been proposed as potential targets for cancer treatment (30-34). As stimulation of the β_2 -adrenergic receptor



Figure 6. The PP1 inhibitor, calyculin A, antagonized β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. pERK levels following treatment with terbutaline (1 μ M) for 10 min with or without pretreatment with the PP1 inhibitor calyculin A (10 nM) for 30 min are presented for (A) MDA-MB-231 and (B) MDA-MB-468 cells. Calyculin A inhibited β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation in both cell lines. Representative Western blot bands for pERK and tERK are presented. Two replicate bands are presented for certain treatment groups. pERK band intensities were normalized to tERK and presented as a percentage of saline + 0.1% DMSO-treated control cells. DMSO (0.1%) was the final dilution of the solvent of calyculin A and was used as the control for pretreatment with calyculin A. Data are presented as the mean ± standard error of the mean, n=4-5. *P<0.05 vs. control, *P<0.05 vs. Ter. PP, protein phosphatase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; tERK, total ERK; DMSO, dimethyl sulfoxide; C, control; Ter, terbutaline; CALY, calyculin A.



Figure 7. β_2 -adrenergic receptor stimulation reduced the level of the inactive form of PP1 in MDA-MB-231 cells. Representative western blot bands are presented for pPP1, tPP1 and GAPDH following treatment of MDA-MB 231 cells with terbutaline (1 μ M) for 2-30 min, and for pPP1, tPP1, pPP, tPP2 and GAPDH following treatment of MDA-MB 231 cells with terbutaline (1 μ M) for 10 min. Terbutaline treatment reduced the level of the inactive form of PP1 (pPP1) and had no effect on the levels of tPP1, pPP2 and tPP2. Two replicate bands are presented for treatment groups. pPP1 and pPP2 band intensities were normalized to tPP1 and tPP2, respectively, and tPP1 and tPP2 band intensities were normalized to GAPDH and presented as a percentage of saline-treated control cells. Data are presented as the mean \pm standard error of the mean, n=4-5. *P<0.05 vs. control cells. PP, protein phosphatase; pPP, phosphorylated PP; tPP, total PP; C, control; Ter, terbutaline.

with endogenous catecholamines may lead to the activation of DUSP1 and PP1, their targets, including p38, c-Jun N-terminal

kinase and ERK1/2, may subsequently be affected. Therefore, as these affected proteins are key components of cancer



Figure 8. β_2 -adrenergic receptor stimulation reduced the level of the inactive form of PP1 in MDA-MB-468 cells. Representative western blot bands are presented for pPP1, tPP1 and GAPDH following treatment of MDA-MB-468 cells with terbutaline (1 μ M) for 10 min. Terbutaline treatment reduced levels of the inactive form of PP1 (pPP1) and had no effect on the expression of tPP1. pPP1 band intensity was normalized to tPP1 and tPP1 band intensity was normalized to GAPDH, and presented as a percentage of saline-treated control cells. Data are presented as the mean ± standard error of the mean, n=4. *P<0.05 vs. control cells. PP, protein phosphatase; pPP, phoshphorylated-PP; tPP, total PP; C, control; Ter, terbutaline.

pathways, the results of the present study, which demonstrated the activation of DUSP1 and PP1 phosphatases following stimulation of β_2 -adrenergic receptors, are important for cancer research. Triple negative breast cancer cells overexpress epidermal growth factor receptor and mutations in KRAS proto-oncogene, B-Raf proto-oncogene and phosphatase and tensin homolog, which lead to high MAPK/ERK1/2 activity and subsequent resistance to therapeutic agents (35). Although the molecular mechanisms have not been completely clarified, previous research has demonstrated that β_2 -adrenergic receptor stimulation mediates pERK1/2 dephosphorylation and inactivation in MDA-MB-231 cells (16,17). Therefore, it is important to investigate the activation of pathways that inactivate MAPK/ERK1/2 in β_2 -adrenergic receptor signaling in these cancer cell lines, including DUSP1 and PP1.

Consistent with previous studies, the present study demonstrated that stimulation of MDA-MB-231 and MDA-MB-468 cells with β_2 -adrenergic receptor agonists resulted in the dephosphorylation of basal pERK1/2 (9,16,17). In addition, the results of the current study indicated that β_2 -adrenergic receptor-mediated dephosphorylation of ERK1/2 was associated with the activity of the protein phosphatases DUSP1 and PP1; DUSP1 and PP1 inhibitors antagonized β_2 -adrenergic receptor-mediated dephosphorylation of ERK1/2. The treatment of MDA-MB-231 and MDA-MB-468 cells with terbutaline increased the protein expression levels of DUSP1 and enhanced the levels of the active form of PP1. Furthermore, reducing the expression of DUSP1 or PP1 reduced β_2 -adrenergic receptor-mediated dephosphorylation of pERK1/2.

The present study investigated the activities of enzymes that may cause pERK1/2 dephosphorylation during β_2 -adrenergic receptor stimulation. Two different types of enzymes, serine/threonine kinases and phosphatases, primarily regulate ERK1/2 phosphorylation status. In particular, pERK1/2 dephosphorylation has been reported to be tightly regulated by DUSP1/6 (22,23,36). Therefore, the activation of DUSP1/6 following β_2 -adrenergic receptor stimulation may trigger the dephosphorylation of pERK1/2. Swingle et al (37) identified a small inhibitor molecule of DUSP1/6, BCI, which directly binds to these phosphatases to inhibit DUSP1 and DUSP6 with IC₅₀ values of 11.5 \pm 2.8 and 12.3 \pm 4.0 μ M, respectively. The results of the current study demonstrated that 10 μ M BCI completely reversed terbutaline-mediated dephosphorylation of pERK1/2, therefore indicating that the β_2 -adrenergic receptor may mediate the activation of DUSP1/6. Several studies have reported that DUSP1 is a labile and inducible enzyme that is primarily localized in the nucleus (22). Price *et al* (19) demonstrated that β -adrenergic receptor stimulation led to a rapid increase in DUSP1 mRNA and protein levels. Considering this, the present study measured the levels of DUSP1 protein in MDA-MB-231 and MDA-MB-468 cells following stimulation with terbutaline and observed a rapid increase in cellular DUSP1 protein levels within 5-30 min of terbutaline stimulation. These results indicate that β_2 -adrenergic receptor stimulation increases the level of DUSP1 protein. Wu et al (36) reported an association between DUSP1 induction and ERK1/2 inhibition. The current study also observed a clear association between the induction of DUSP1 expression and ERK1/2 dephosphorylation following β_2 -adrenergic receptor stimulation. Therefore, it was predicted that inhibiting the expression level of DUSP1 may lead to a decline in terbutaline-mediated pERK1/2 dephosphorylation. In the current study, downregulation of DUSP1 expression by siRNA transfection led to a decline in terbutaline-mediated pERK1/2 dephosphorylation. These results also indicate an association between β_2 -adrenergic receptor stimulation and the expression and activation of DUSP1.

DUSP6, which is generally localized in the cytosol as a phosphatase, is primarily responsible for ERK1/2 dephosphorylation (22,23). Although our experiments did not demonstrate a significant alteration in the level of DUSP6 protein following terbutaline stimulation, this does not eliminate a potential role for DUSP6 protein in β_2 -adrenergic receptor-mediated ERK1/2 dephosphorylation. The expression and activity of DUSPs may be involved in terbutaline-mediated dephosphorylation of ERK1/2. BCI inhibits both DUSP1 and DUSP6, and, in the present study, it completely reversed the dephosphorylation of ERK1/2. Further studies are therefore required to clarify the influence of β_2 -adrenergic receptor stimulation on DUSP6 activity.

It is established that ERK1/2 phosphorylation is also regulated by the serine/threonine phosphatases PP1 and PP2 (25). Calyculin A is a PP1 and PP2 inhibitor with IC₅₀ values of 0.4 and 0.25 nM, respectively (37). At concentrations of 50-100 nM, calyculin A is cytotoxic, killing the majority of human cell types, while at 10 nM it is suitable for treating cells (37). Therefore, if there is an association between PP1/2



Figure 9. Downregulation of PP1 reduced β_2 -adrenergic receptor-mediated pERK1/2 dephosphorylation. (A) Expression levels of PP1, GAPDH, pERK and tERK following transfection with control siRNA (1 μ M) or PP1 siRNA (1 μ M), and levels of PP1, GAPDH, pERK and tERK following treatment of control or PP1 siRNA-transfected cells with terbutaline (0.1 μ M) or saline for 5-10 min, for (A) MDA-MB-231 and (B) MDA-MB-468 cells. Transfection of cells with PP1 siRNA downregulated PP1 in both cell. Downregulation of PP1 reduced terbutaline-mediated dephosphorylation of pERK. Band intensities were normalized to GAPDH for PP1 and tERK for pERK. Results in the bar graph indicate the percentage of dephosphorylated ERK following terbutaline treatment in control and PP1 siRNA-transfected MDA-MB-231 and MDA-MB-468 cells, and are presented as a percentage of saline-treated control cells. Data are presented as the mean ± standard error of the mean, n=4. *P<0.05 vs. control cells. PP, protein phosphatase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; tERK, total ERK; siRNA, small interfering RNA; csiRNA, control siRNA; siPP1, siRNA targeting PP1; C, control; Ter, terbutaline.

activity and β_2 -adrenergic receptor-mediated dephosphorylation of pERK1/2, calyculin A should influence the response. In the present study, treatment of cells with 10 nM calyculin A antagonized the terbutaline-mediated dephosphorylation of pERK1/2. Following the use of an enzyme inhibitor to block the response as a pharmacological approach, the present study also determined the activity of these enzymes in β_2 -adrenergic receptor-mediated ERK1/2 dephosphorylation by measuring pPP1 and pPP2 levels (inactivated forms of PP1 and PP2) using western blot analysis. Stimulation of the cells with terbutaline (2, 5 and 10 min) significantly diminished pPP1 in the cells, while pPP2 levels were not affected, indicating that the β_2 -adrenergic receptor may mediate the activation of PP1. However, the same response for pPP1 levels was not observed when the cells were stimulated with terbutaline for 30 min. These results clearly indicate the presence of a similar pattern between terbutaline-mediated pERK1/2 and pPP1 dephosphorylation during the 30 min stimulation. As β_2 -adrenergic receptor stimulation led to increased DUSP1 expression, rather than phosphorylation status, tPP1/tPP2 expression levels were also measured, and the results demonstrated that tPP1 and tPP2 levels were not altered following terbutaline stimulation. This indicates that the β_2 -adrenergic receptor-mediated decline in the inactive form of PP1 was not due to altered tPP1

protein expression level. If the activation of the β_2 -adrenergic receptor activates PP1 then downregulation of PP1 expression should reduce the β_2 -adrenergic receptor-mediated activation of PP1 and, consequently, the dephosphorylation of ERK1/2. To test this hypothesis, in the present study, cells were transfected with PP1 siRNA to downregulate its cellular expression, which reduced terbutaline-mediated pERK1/2 dephosphorylation. These results indicate that PP1 activity may be important in β_2 -adrenergic receptor-mediated ERK1/2 dephosphorylation. Consistent with the results of the current study, Chruscinski *et al* (20) investigated the phosphorylation status of several signaling proteins in mouse embryonic fibroblast cells and showed that β -adrenergic receptor stimulation resulted in the dephosphorylation and activation of PP1.

In the current study, β_2 -adrenergic receptor-mediated dephosphorylation was investigated in MDA-MB-231 and MDA-MB-468 triple negative breast cancer cell lines. These cells express high levels of the β_2 -adrenergic receptor (12) and high ERK1/2 activity, with high pERK1/2 levels (21). Other breast cancer cell lines differ in terms of the expression of HER2, ER and PR, and these cell lines possess different properties compared with triple negative breast cancer cell lines. These differences may result in variability with regards to the mechanism of β_2 -adrenergic receptor-mediated dephosphorylation of ERK1/2. Therefore, to reduce variability, the present study performed experiments in only two different, triple negative breast cancer cell lines. Further studies are required to investigate the associations among the β_2 -adrenergic receptor, pERK1/2, DUSP1 and PP1 in breast cancer cells other than the triple negative type. In addition, further studies, such as directly measuring the activity of DUSP1 and PP1 with β_2 -adrenergic receptor stimulation in breast cancer cell lines, should be performed to further confirm these results, as only western blot analysis was performed in the present study to identify the association between the β_2 -adrenergic receptor and DUSP1 and PP1.

In conclusion, the present study demonstrated that β_2 -adrenergic receptor stimulation led to the dephosphorylation of ERK1/2, which was inhibited by the DUSP1 inhibitor BCI and the PP1 inhibitor calvculin A, and increased DUSP1 expression and PP1 activity. Furthermore, downregulation of DUSP1 and PP1 expression reduced terbutaline-mediated pERK1/2 dephosphorylation. Therefore, the results of the present study demonstrated that DUSP1 and PP1 may have important roles in β_2 -adrenergic receptor-mediated ERK1/2 dephosphorylation in MDA-MB-231 and MDA-MB-468 breast cancer cells. β_2 -adrenergic receptor-mediated inactivation of ERK1/2 or activation of DUSP1 and PP1 can dephosphorylate and inactivate some malignant signaling molecules and may effect cancer progression. The consequences of this action of the β_2 -adrenergic receptor should be examined in preclinical and clinical studies, particularly when β_2 -adrenergic blockers are used in patients with triple negative breast cancer.

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