〈Original article〉

Inhibition of PP2A in MCF-7 cells leads to hormone-independent growth.

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Summary Aromatase inhibitors (AIs) are the most effective endocrine therapy for postmenopausal women with hormone receptor positive (HR+) breast cancer. However, the efficacy of the treatment is often limited by the onset of AIs resistance due to phosphorylation of oestrogen receptor (ER)α serine 167 (S167). Long-term oestrogen-deprived (LTED) cells, AIs-resistant breast cancer models, are hypersensitive to IGF-1, which coincides with elevated levels of ERα phosphorylated on S167. Effects of rapamycin disappeared by using a protein phosphatase inhibitor cocktail, PhosSTOP (PS), and ERα S167 phosphorylation was restored. Moreover, resistance to oestrogen depletion was allowed by protein phosphatase Type 1 and 2A inhibitor O.A. Our results suggest that the powerful inhibitory effect of rapamycin on phosphorylation was attributed to the involvement of a phosphatase, and the possibility that PP2A affects ERα S167 phosphorylation and oestradiol (E2)-independent growth.

Key words: Protein Phosphatase Type2A, breast cancer, Estrogen, Estrogen receptor

1. Introduction

Endocrine therapy is the most effective systemic treatment for patients with HR+ breast cancer. Oestrogen deprivation therapy with nonsteroidal third-generation AIs is more effective than tamoxifen for postmenopausal women with HR+ advanced breast cancer[1]. Unfortunately, the efficacy of the treatment is often limited by the onset of resistance, which is almost inevitable in patients with advanced disease. Occasionally, AIs treatment resistant ERα positive breast cancers are observed in the course of endocrine therapy. The findings of the previous study revealed crosstalk between the IGF-I signalling pathway and ERα in breast cancer cells. Abnormally activation of ERα is dependent on phosphorylation[2] of the serine residues S104, S106, S118, and S167[3] located in the amino terminal A/B domain of ERα[4].

Small-molecule inhibition and targeted knockdown of S6 kinase 1 (S6K1) blocked IGF-1 induced phosphorylation of ERα at residue S167. Inhibition of S6K1 kinase activity consequently ablated IGF-1 stimulated S6K1/ERα association and subsequent ERα target gene transcription[5].

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Interestingly, phosphorylation of ERα S167 by S6K1 is implicated in breast cancers refractory to endocrine therapy. As mentioned above, previous studies have shown that IGF-1 signalling increases phosphorylation of S167, which activates ERα. However, it is not clear whether dephosphorylation of this serine residue is as important as its phosphorylation. In this study, we have examined the effect of a phosphatase inhibitor on proliferation of an ERα (+) breast cancer cell line (MCF-7).

2. Materials and Methods

1. Reagents

MCF-7 cells (a human ER-positive breast cancer cell line) were obtained from the American Type Culture Collection (Rockville, MD). IGF-1 was purchased from Sigma (St. Louis, MO). Antibodies against Akt, phosphorylated Akt, ERα, phosphorylated ERα S167, and beta-actin were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against phosphorylated ERα S118 was purchased from Cell Signaling Technology (Danvers MA), respectively.

2. Cell culture

MCF7 cells were maintained in Roswell Park Memorial Institute medium (RPMI) (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum and 1% of penicillin/streptomycin at 37°C in a 95% air-5% CO^2 humidified atmosphere incubator. Cells treated with 17 beta-oestradiol (E2) and IGF-1 (Sigma, St. Louis, USA) were cultured in phenol red-free RPMI medium supplemented with 10% dextran coated charcoal (DCC) treated fetal bovine serum (Nichirei Biosciences Inc., Tokyo JP) and 1% penicillin/streptomycin. LTED cells modelling resistance to an AI were derived from a parental cell line by long-term culture in the presence of RPMI1640 medium containing 10% DCC serum, as described previously[6]. Cell viability of cultured cells was quantified using the Cell Counting Kit 8 (Dojindo Molecular Technologies, Maryland, USA).

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3. Western blot analysis

The samples (5 μg protein/lane) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, UK). The membrane was pre-incubated with ImmunoBlock (DS Pharma Biomedical Co., Ltd. Japan) at room temperature for 30 min, and then incubated overnight with antibodies against ERα, phosphorylated ERα S167, and ERα S118, beta-actin rabbit polyclonal antibody at 4°C. The membrane was subsequently washed with TBS-T buffer (20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% Tween-20) and incubated with a horseradish peroxidase-labelled secondary anti-rabbit (Bio-Rad Laboratories, Inc., Hercules, CA) or anti-mouse (MBL, Nagoya, Japan) IgG antibody for 1 h. After washing the membrane with TBS-T buffer, the immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA). Intensity of the chemiluminescence of specific bands was digitized using Cool Saver (ATTO, Tokyo, Japan) and quantified. All antibodies were diluted with Can Get Signal Immunoreaction Enhancer Solution (Toyobo, Inc., Osaka, Japan).

3. Results

1. Inhibition of PP2A enhanced ERα S167 phosphorylation.

MCF-7 cells have served as a model for the study of oestrogen response in vitro. In vitro studies using oestrogen withdrawal or chronic exposure to anti-oestrogen have led to the isolation of hormone-resistant variants of MCF-7 cells. Oestrogen withdrawal initially slows cell growth; however, growth eventually resumes. LTED cells have been generated by several laboratories. LTED cell lines have been used as AIs-resistant breast cancer models. In agreement with previously published studies, LTED cells are hypersensitive to IGF-1[5]. Phosphorylation of ERα S167 in LTED cells was induced by long term oestrogen deprivation as MCF-7 parental cells; however, phosphorylation of

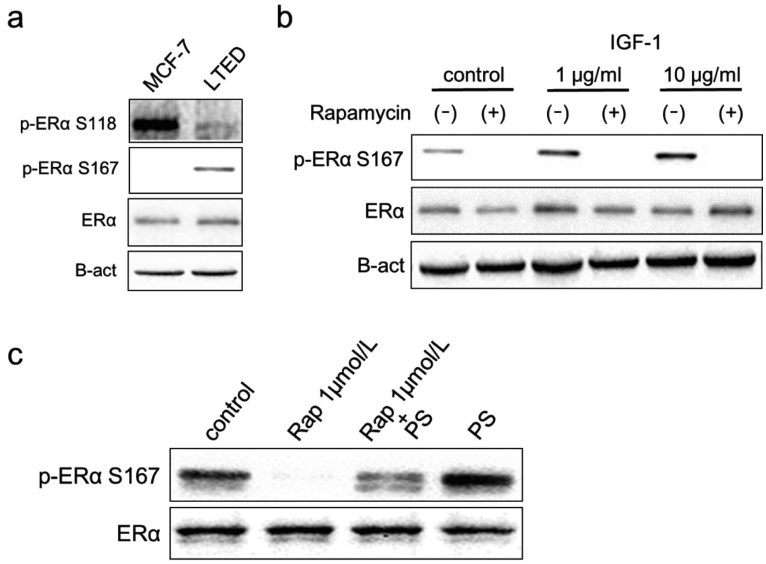

Fig. 1 Western Blot analysis of MCF-7 and LTED.
a; ERα and phosphorylated ERα S118/S167, beta-actin in MCF-7 and LTED cells.
b; Effects of rapamycin and response to IGF-1. LTED cells were incubated in the absence or presence of rapamycin (1μM) and IGF-1 for 1 h, as indicated, and were then analyses with immunoblotting of membranes from two separate gels.
c; LTED cells were incubated with rapamycin (1μM) and PS for 1 hr. Cells were lyzed and 5 μg total protein was resolved by electrophoresis on a 10% SDS-PAGE gel. Beta-actin and ERα protein was used as a loading control.

S118 was compromised in LTED cells (Fig 1a). ERα S167 phosphorylation in LTED cells is promoted by mTOR activation. Next, we examined the effect of rapamycin, a known dual inhibitor of IGF-1 and mTOR, on ERα S167 phosphorylation. Cells were treated with rapamycin for 1 h. IGF-1 concentration-dependent ERα S167 phosphorylation was strongly reduced by rapamycin treatment (Fig 1b). The powerful inhibitory effect of rapamycin on phosphorylation was attributed to the involvement of a phosphatase, protein phosphatase-2A (PP2A). Effects of rapamycin disappeared by using a protein phosphatase inhibitor cocktail, PhosSTOP (PS), and ERα S167 phosphorylation was restored (Fig 1c).

2. PP2A inhibition induces oestrogen-independent cell growth.

Oestrogen deprivation to induce cell death on Oestrogen-dependent breast cancer MCF-7. To explore the role of PP2A in this process, we determined whether PP2A inhibition by O.A could induce oestrogen-independent growth. Previous

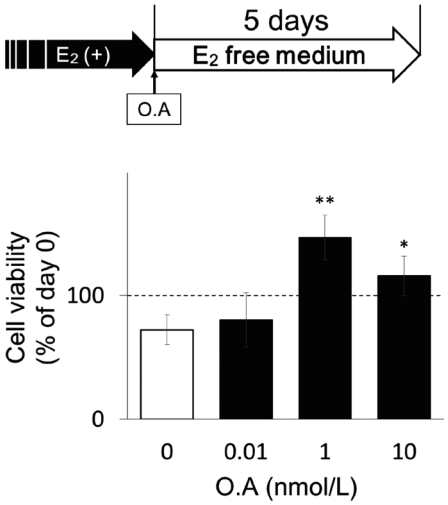

Fig. 2 Oestrogen deprivation resistance to PP2A inhibitor in MCF-7.
MCF-7 cells to various concentrations (0.01-10nmol/L) of O.A in phenol red-free RPMI 1640 supplemented with 10% DDC fetal bovine serum for 5 days (n=4).
*Control versus 10nmol/L O.A ($P < 0.01$).
**Control versus 1nmol/L O.A ($P < 0.001$).

studies have demonstrated a concentration-dependent effect of O.A on PP2A inhibition. MCF-7 cells in oestrogen-depleted medium were treated with the PP2A inhibitor O.A (10^{-11} – 10^{-8} mol/L) for 5 days. Resistance to oestrogen depletion was inhibited by O.A in a concentration-dependent manner (Fig. 2).

4. Discussion

ERα S167 phosphorylation changes significantly within a short period, as shown in Fig.1. Phosphorylation level is determined by the activities and balance of protein kinases and phosphatases. Our results indicate that phosphatases play an important role in the regulation of ERα S167 phosphorylation. O.A is a polyether derivative of a 38-carbon fatty acid and is the causative agent of diarrheic shellfish poisoning. O.A inhibited protein phosphatase-1 (IC50=10-20nmol/L) but was slightly less active against PP2A (IC50=0.1-0.2nmol/L) [7]. Our data show the effect of phosphatase inhibitor on the proliferation of MCF-7 cells. As a point of interest, results with the highest effective concentration of O.A (1nmol/L) suggest that PP2A is involved in the acquisition of AIs resistance.

The phosphorylation of ERα S167 is an important modification required for the acquisition of AIs resistance in breast cancer cells. Therefore, IGF-1 signalling pathway-dependent activation of ERα was assumed to be the reason for AIs resistance[8], and the role of S6K1 has been elucidated in previous studies[5, 9]. PP2A is a key tumor suppressor that regulates signalling pathways relevant to many human cancers[10, 11]. Previous studies suggest that PP2A dephosphorylates and inactivates S6K through its interaction with the N-terminus of S6K1[12]. However, the relationship between oestrogen depletion and expression of PP2A involvement has not been explored. Additionally, dephosphorylation mechanism of S6K1 and its relation to ERα S167 phosphorylation is not clearly understood.

PP2A is a ubiquitously expressed member of a large protein phosphatase family, involved in the regulation of cell proliferation, cell differentiation, RNA transcription, DNA repair, and apoptosis[13-15]. PP2A inhibits major signal transduction pathways, including the phosphatidylinositol 3-kinase/AKT and mitogen-activated protein kinase pathways. Our results suggest that PP2A inhibition affects ERα S167 phosphorylation and E2-independent growth in MCF-7 cells. Future studies should investigate the relationship between E2 depletion and PP2A expression.

References

1. Ellis M: Overcoming endocrine therapy resistance by signal transduction inhibition. Oncologist, 9 Suppl 3: 20-6, 2004.
2. Orti E, Bodwell JE, Munck A: Phosphorylation of steroid hormone receptors. Endocr Rev, 13: 105-28, 1992.
3. Le Goff P, Montano MM, Schodin DJ, et al.: Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J Biol Chem, 269: 4458-66, 1994.
4. Arnold SF, Obourn JD, Jaffe H, et al.: Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. Mol Endocrinol. 8: 1208-14, 1994.
5. Huderson BP, Duplessis TT, Williams CC, et al.: Stable inhibition of specific estrogen receptor alpha (ERalpha) phosphorylation confers increased growth, migration/invasion, and disruption of estradiol signaling in MCF-7 breast cancer cells. Endocrinology, 153: 4144-59, 2012.
6. Martin LA, Farmer I, Johnston SR, Ali S, et al.: Enhanced estrogen receptor (ER) alpha, ERBB2, and MAPK signal transduction pathways operate during the adaptation of MCF-7 cells to long term estrogen deprivation. J Biol Chem, 278: 30458-68, 2003.
7. Haystead TA, Sim AT, Carling D, et al.: Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature, 337: 78-81, 1989.
8. Johnston SR: New strategies in estrogen receptor-positive breast cancer. Clin Cancer Res, 16: 1979-87, 2010.
9. Becker MA, Ibrahim YH, Cui X, et al.: The IGF pathway regulates ERalpha through a S6K1-dependent mechanism in breast cancer cells. Mol Endocrinol, 25: 516-28, 2011.
10. Mumby, M: PP2A: unveiling a reluctant tumor

suppressor. Cell, 130, 21-4, 2007.
11. Westermarck J, & Hahn WC: Multiple pathways regulated by the tumor suppressor PP2A in transformation. Trends Mol Med, 14: 152-60, 2008.
12. Peterson RT, Desai BN, Hardwick JS, et al.: Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc Natl Acad Sci U S A, 96: 4438-42, 1999.
13. Kong M, Fox CJ, Mu J, et al.: The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. Science, 306: 695-8, 2004.
14. Sontag E: Protein phosphatase 2A: the Trojan Horse of cellular signaling. Cell Signal, 13: 7-16, 2001.
15. Janssens V, Goris J: Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. Biochem J, 353: 417-39, 2001.