Phorbol myristate acetate suppresses breast cancer cell growth via down-regulation of P-Rex1 expression

Dear Editor,

P-Rex1 is a Rac-selective guanine nucleotide exchange factor (GEF) that is synergistically activated by G-protein coupled receptors and receptor tyrosine kinases (Welch et al., 2002). We previously reported that aberrantly upregulated P-Rex1 promotes prostate cancer metastasis by activating Rac1 signals (Qin et al., 2009). P-Rex1 is also highly overexpressed in estrogen receptor-positive and ErbB2-overexpressing human luminal breast tumors, which correlates with the aggressiveness of human breast cancer and poor outcome in breast cancer patients (Montero et al., 2011; Sosa et al., 2010). Silence of endogenous P-Rex1 blocks breast cancer cell proliferation, tumorigenesis, and motility (Montero et al., 2011; Sosa et al., 2010). Therefore, P-Rex1 is an important mediator in cancer progression and could be a potential therapeutic target.

Protein kinase C (PKC), a family of serine-threonine kinases, has been implicated in breast cancer progression (Urtreger et al., 2012). PKC isoforms are classified into conventional (α, β, and γ), novel (δ, ε, η, and θ), and atypical (ζ and λ) PKCs. Expression profiles of PKC isoforms vary among different breast cancer cell lines (Urteger et al., 2012). Phorbol 12-myristate 13-acetate (PMA), a structural homolog of diacylglycerol (DAG), activates conventional and novel PKCs. PMA treatment induces breast cancer cell growth arrest via sustained up-regulation of the cell-cycle inhibitor p21 (WAF1/CIP1) (Barboule et al., 1999; Fortino et al., 2008). Interestingly, Rac1 was reported to be overexpressed or hyperactive in breast cancer tissues (Schnelzer et al., 2000) and hyperactivity of Rac1 suppressed p21 (WAF1/CIP1) expression in cancer cells (Knight-Krajewski et al., 2004). Since P-Rex1 functions as a Rac1 activator in cancer cells (Qin et al., 2009; Sosa et al., 2010), the purpose of the present study was to determine the role of P-Rex1 in PMA inhibition of breast cancer cell growth.

Both MCF-7 and BT-474 cell lines, derived from human luminal breast cancers, are ER-positive and highly express P-Rex1 (Sosa et al., 2010). MCF-7 cells are also ErbB2-positive whereas BT-474 cells are ErbB2-overexpressed. Thus, these two cell lines were chosen for our studies.

Western blot analysis showed that the P-Rex1 protein expression level in BT-474 cells is 4.5-fold higher than that in MCF-7 cells (Fig. 1A). Thirty hours treatment with PMA caused a concentration-dependent decrease in P-Rex1 protein levels in both MCF-7 and BT-474 cells with a maximum reduction of 87.2% ± 1.1% and 57.0% ± 8.6%, respectively, at a concentration of 10 ng/mL PMA (Fig. 1B). PMA also significantly attenuated growth of both MCF-7 and BT-474 cells in a concentration-dependent manner with an inhibition of 77.8% ± 12.4% and 50.6% ± 3.7%, respectively, at 10 ng/mL PMA (Fig. 1C). Interestingly, PMA-induced inhibition of cell growth is correlated to the degree of P-Rex1 down-regulation in MCF-7 and BT-474 cells. Thus, a recovery assay was performed to determine whether PMA inhibition of breast cancer cell growth is P-Rex1 dependent. As shown in Fig. 1D inset, expression of recombinant P-Rex1 restored the P-Rex1 expression level in PMA-treated MCF-7 cells. PMA treatment dramatically reduced the growth of control MCF-7 cells but not cells transfected with P-Rex1. Expression of recombinant P-Rex1 had little effect on MCF-7 cell growth in the absence of PMA but completely restored cell growth in the presence of PMA (Fig. 1D). Although transfection of recombinant P-Rex1 plasmid only slightly increased P-Rex1 protein level in untreated BT-474 cells, it partially restored the P-Rex1 protein expression in PMA-treated BT-474 cells (Fig. 1E, inset). More importantly, expression of recombinant P-Rex1 increased PMA-treated BT-474 cell growth by 1.7-fold, which equals 70% of untreated control cells (Fig. 1E).

Hyperactivated ErbB receptor signaling has been frequently characterized in breast carcinomas (Hynes and Lane, 2005). P-Rex1 is an essential mediator of ErbB signaling in breast cancer (Sosa et al., 2010). Thus, we silenced endogenous P-Rex1 expression in MCF-7 and BT-474 cells by over 80% using P-Rex1 specific siRNA (Fig. 1F and 1G, inset). Treatment with heregulin (100 ng/mL), an ErbB activating ligand, increased proliferation of MCF-7 (Fig. 1F) and BT-474 (Fig. 1G) cells transfected with control siRNA by 1.8-fold and 2.1-fold respectively, as indicated by BrdU incorporation assay. However, this stimulatory effect was significantly reduced in cells transfected with P-Rex1 siRNA (Fig. 1F and 1G).
Together, our data suggest that P-Rex1 functions as a key molecule in breast cancer cell growth and that down-regulation of P-Rex1 contributes to PMA suppression of MCF-7 and BT-474 cell growth.

PMA mimics DAG in the cellular membrane to activate PKC. Treatment with PMA (10 ng/mL) significantly increased PKC kinase activity in both MCF-7 and BT-474 cells, which was completely blocked by pre-treatment with the general PKC inhibitor Gö6983 (2 µmol/L) (Fig. S1A). Western blot assay showed that both MCF-7 and BT-474 cells express PMA-sensitive conventional PKC isoforms (α and β) and novel PKC isoforms (δ, ε and η). MCF-7 cells also express...
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In summary, our study is the first to use a small molecule, PMA, to target P-Rex1 expression levels to suppress breast cancer cell growth. PMA itself has both oncogenic and antitumorigenic properties by direct or indirect modulation of various cellular targets (Griner and Kazanietz, 2007). Previous studies suggest that induction of the cell-cycle inhibitor p21 (WAF1/CIP1) is involved in connecting the PMA-activated PKC signaling pathways to the breast cancer cell cycle regulatory machinery, leading to cell growth arrest (Barboule et al., 1999; Fortino et al., 2008). Interestingly, Rac1 was reported to be overexpressed or hyperactive in breast cancer tissues and hyperactivity of Rac1 suppressed p21 (WAF1/CIP1) expression in cancer cells. Since P-Rex1 functions as a Rac1 activator in cancer cells, PMA-down-regulation of P-Rex1 expression should result in reduction of Rac1 activity, leading to increased expression of p21 (WAF1/CIP1). Thus, our study provides a potential molecular mechanism underlying PMA suppression of breast cancer cell growth.

Our studies further showed that active PKCε, but not the other PMA-sensitive PKC isomers, down-regulates P-Rex1 expression and suppresses breast cancer cell growth. PKCε has a unique role in regulating cell-signaling pathways in cancer (Griner et al., 2007; Urtreger et al., 2012). Elevated PKCε levels were correlated with breast cancer aggressiveness (Pan et al., 2005). Our study presents the first evidence suggesting that the PKCε/P-Rex1 pathway may be an attractive new target for therapeutic intervention, which provides an additional approach for improving the current treatment of breast cancer. For example, estrogen receptor-targeted therapies have significantly reduced breast cancer mortality. However, resistance generally emerges because various growth factor receptors such as ErbB2 can transactivate estrogen receptors in an estrogen-independent manner, contributing to tumor growth. Trastuzumab, a monoclonal ErbB2 antibody, has significant clinical benefit for patients with ErbB2-elevated breast tumors (Smith et al., 2007). However, patients may also develop resistance within 1 year of treatment. A common feature of the possible mechanisms of resistance is Rac1 activation and inactivation of Rac1 reduces Trastuzumab resistance in breast cancer cells (Zhao et al., 2011). P-Rex1 is highly expressed in human breast cancers with high ErbB2 and estrogen receptor expression and functions as a Rac-specific activator at a convergence point downstream of Rac1. Thus, it has been suggested as an attractive therapeutic target (Sosa et al., 2010). Understanding PKCε-dependent P-Rex1 down-regulation may provide a novel strategy for development of chemotherapeutic agents for P-Rex1-overexpressing breast cancer patients that develop resistance to anti-estrogen and/or anti-ErbB2 therapies.

FOOTNOTES

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Figure 2. PKCε activation contributes to PMA suppression of breast cancer cell growth through P-Rex1 down-regulation. The general PKC inhibitor Gö6983 (2 µmol/L), but not the conventional PKC isoform inhibitor Gö6976 (100 nmol/L), attenuated PMA (10 ng/mL) suppression of P-Rex1 expression (A) and breast cancer cell growth (B). (A) Western blot analysis of P-Rex1 expression. Data are means ± SEM (n = 3) with *P < 0.01 compared to cells without PMA treatment. (B) Cell growth assay. Cells were cultured in the presence or absence of PMA for 48 h (MCF-7) or 72 h (BT-474). Data are means ± SEM (n = 5 of duplicates) with *P < 0.01 compared to cells without PMA treatment. (C) Expression of PKCε constitutively active form (CAT), but not PKCε wild-type (WT), PKCδ or PKCη WT and CAT mutant, down-regulated P-Rex1 expression in MCF-7 and BT-474 cells. Data are means ± SEM (n = 3) with *P < 0.01 compared to cells transfected with vector. (D) Gö6983 (2 µmol/L) attenuated PKCε CAT-induced down-regulation of P-Rex1 expression in MCF-7 cells. (E) Expression of PKCε CAT, but not its WT, suppressed MCF-7 cell growth, which is blocked by treatment with Gö6983 (2 µmol/L). Data are means ± SEM (n = 3 of duplicates) with *P < 0.01 compared to cells transfected with vector. Insets: Representative Western blot images of P-Rex1, PKCε, and β-actin protein expression in breast cancer cells.
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Chuu-Yun A. Wong, Haihong Jiang, Peter W. Abel, Margaret A. Scofield, Yan Xie, Taotao Wei, and Yaping Tu declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by the any of the authors.

Chuu-Yun A. Wong 1, Haihong Jiang 1, Peter W. Abel 1, Margaret A. Scofield 1, Yan Xie 1, Taotao Wei 2, Yaping Tu 1

1 Department of Pharmacology, Creighton University School of Medicine, Omaha, NE 68178, USA
2 National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Correspondence: weitt@moon.ibp.ac.cn (T. Wei), Yat60399@creighton.edu (Y. Tu)

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