Protein Kinase Cα Modulates Estrogen-Receptor-Dependent Transcription and Proliferation in Endometrial Cancer Cells

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Received 10 May 2013; Accepted 31 May 2013

Academic Editor: Donghai Dai

Endometrial cancer is the most common invasive gynecologic malignancy in developed countries. The most prevalent endometrioid tumors are linked to excessive estrogen exposure and hyperplasia. However, molecular mechanisms and signaling pathways underlying their etiology and pathophysiology remain poorly understood. We have shown that protein kinase Cα (PKCα) is aberrantly expressed in endometrioid tumors and is an important mediator of endometrial cancer cell survival, proliferation, and invasion. In this study, we demonstrate that expression of active, myristoylated PKCα conferred ligand-independent activation of estrogen-receptor-(ER-) dependent promoters and enhanced responses to estrogen. Conversely, knockdown of PKCα reduced ER-dependent gene expression and inhibited estrogen-induced proliferation of endometrial cancer cells. The ability of PKCα to potentiate estrogen activation of ER-dependent transcription was attenuated by inhibitors of phosphoinositide 3-kinase (PI3K) and Akt. Evidence suggests that PKCα and estrogen signal transduction pathways functionally interact, to modulate ER-dependent growth and transcription. Thus, PKCα signaling, via PI3K/Akt, may be a critical element of the hyperestrogenic environment and activation of ER that is thought to underlie the development of estrogen-dependent endometrial hyperplasia and malignancy. PKCα-dependent pathways may provide much needed prognostic markers of aggressive disease and novel therapeutic targets in ER positive tumors.

1. Introduction

Endometrial cancer is the most common invasive gynecological malignancy in the United States, accounting for 45,000 new cancer cases and over 7,500 deaths annually [1]. However, molecular mechanisms underlying its etiology and pathophysiology are poorly understood. Endometrial carcinomas are derived from glandular epithelium and typically divided into two subtypes based on clinical, histological, and molecular characteristics [2, 3]. Type I tumors, comprising 80% of cases, are generally well or moderately differentiated with endometrioid morphology and are associated with chronic unopposed estrogen exposure and hyperplasia. By contrast, type II tumors are more heterogeneous, poorly differentiated and may be estrogen independent, arising in a background of atrophic endometrium [2, 4]. The prevalence of advanced stage, high-grade tumors, of both types, with recurrent metastatic disease is increasing [5, 6]. Such cancers typically have a poorer prognosis and are refractory to current therapeutic regimens [7].

Endometrioid tumors retain expression of estrogen (ER) and progesterone (PR) receptors [8], and estrogen is a critical regulator of endometrial proliferation [9, 10]. Indeed, the majority of endometrial cancers are thought to arise due to unopposed estrogen action leading to hyperplasia and malignant transformation [2, 11]. However, our understanding of the molecular mechanisms underlying the pathophysiology of endometrial cancer lags far behind that of other hormone-dependent malignancies such as breast, prostate and ovarian cancer [2, 8, 12, 13].
The protein kinase C (PKC) family has been implicated in the regulation of numerous signal transduction pathways, modulating cell growth, differentiation, and survival [14–16]. In endometrial cancer cells and primary endometrial epithelium, expression of PKCα is increased in response to treatment with estrogen and tamoxifen and may underlie the proliferative actions of these agents in the endometrium [17, 18]. We have previously shown that PKCα is aberrantly expressed in human endometrial tumors [19, 20] and is a critical regulator of endometrial cancer cell survival, proliferation, transformation, invasion, and response to chemotherapy [21, 22]. In addition, we demonstrated that knockdown of PKCα inhibits growth of estrogen-dependent endometrial cancers in an in vivo model [20].

In this study, we present evidence that, in type I endometrial cancer cells, PKCα induces hormone-independent activation of ER, potentiates estrogen transcriptional responses, and regulates estrogen-dependent proliferation and gene expression. Thus, PKCα signaling may be a critical element of the supraphysiologic activation of ER thought to underlie the development of endometrial hyperplasia and malignancy.

2. Materials and Methods

2.1. Cell Lines. Ishikawa and HEC-50 endometrial carcinoma cells were a generous gift from Dr. Leslie (University of Iowa). Ishikawa cells expressing luciferase (luc) or PKCα shRNAs have been described [21]. Unless stated otherwise, all cell lines were maintained in 5% CO₂, phenol red free DMEM, supplemented with charcoal stripped 10% fetal bovine serum, 10 units/mL penicillin, 10 μg/mL streptomycin, and 200 μM L-glutamine. Prior to estrogen treatment (100 nM Estradiol, Sigma Aldrich, St. Louis, MO, USA), cells were transferred to phenol red free DMEM containing 1x SR-1 serum replacement (Sigma Aldrich, St. Louis, MO, USA). Cell lines used were authenticated by analysis of DNA microsatellite short tandem repeats (STRs), as described previously [23].

2.2. Cell Proliferation. Cell number and viability were determined from subconfluent cultures using a Vi-Cell Coulter Counter (Beckman-Coulter, Inc., Fullerton, CA, USA) as described in [20].

2.3. Luciferase Reporter Assays. The ERE-luc and pS2-luc promoter reporter constructs have been described in [24–26]. Myristoylated PKCα vector [27] was obtained from Addgene (Cambridge, MA). Cells (2.0 × 10⁵) were transiently transfected with 0.5 μg ERE-Luc or pS2-luc reporter plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturers protocol. 0.5 μg pcMVβ, encoding β-galactosidase under control of the CMV constitutive promoter, was included as a control for transfection efficiency and cell number. Total DNA was kept constant by addition of empty vectors. Promoter activity was determined by Luciferase and β-galactosidase assays, as described in [28].

2.4. RNA Isolation and Quantitative RTPCR. RNA was isolated from 10⁶ cells using a Qiagen RNeasy kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s directions and quantitated using a NanoDrop ND1000 spectrophotometer. Aliquots were evaluated by chromatography using an Agilent RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA) on an Agilent Bioanalyzer 2100 system. cDNAs were prepared using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) as per the manufacturers instructions. The samples were amplified by real-time PCR using iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA) on a Bio-Rad CFX96 C1000 Thermal Cycler using the following conditions: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Negative control RNA samples were not reverse transcribed or did not lack PCR template. Results were analyzed with qbasePLUS software (Bio-Rad Hercules, CA, USA), and changes in expression, relative to β-actin and rpl13a controls, were estimated using the ΔΔCT method [29]. Primer pair sequences (forward and reverse, 5’ to 3’) were as follows: β-Actin: AGCGCTCGCC- TTTGCCGA and GCCCGCGCATATCATC; RPL13A: TACCAGAAGTTTTGCTTAGTGGG and TGCCGG- TTCCGTAAAGCTCAAAG; PRKCA: GCTTCCAGTGC- AAGTTTGC and GCACCCGGGACAGAAAAAGTAA; LTF: ATGGTGTGGTTCTATACGAGGCA and GCCACG-GGATAATAGTGGAT; c-FOS: AAAAAAGAGAATCCGA- AGGAAA and GTCTGTCTCCGCTTGGAGTGTAT; pS2 (TFF1): AGGCGCCAGACAGAGTGTAC and CGT- GCATAATAGTGAGTT; PRKCA: GCTTCCAGTGC- AAGTTTGC and GCACCCGGGACAGAAAAAGTAA; LTF: ATGGTGTGGTTCTATACGAGGCA and GCCACG-GGATAATAGTGGAT; c-FOS: AAAAAAGAGAATCCGA-

2.5. Statistical Analysis. Data were expressed as mean ± standard deviation or standard error of the mean and analyzed using Student’s t-test. P values <0.05 were considered significantly different.

3. Results

To investigate the functional role of PKCα signal transduction in the regulation of ER-dependent transcription, Ishikawa endometrial cancer cells were transiently transfected with a myristoylated PKCα construct (myrPKCα) that is targeted to membranes and thereby rendered constitutively active [21, 27]. As shown in Figure 1, expression of myrPKCα, in the absence of estrogen, resulted in a dose-dependent activation of transcription from a promoter containing 3 copies of a canonical estrogen response element (ERE) fused to luciferase [30]. Treatment of Ishikawa cells with estradiol (E2) increased the activity of the ERE promoter approximately 170-fold (Figure 2(a)). In the presence of activated myrPKCα, E2-stimulated ERE promoter activity was further increased over 170-fold. Thus, PKCα induced hormone-independent activity of an ERE and potentiated the effect of estrogen. Similar results were obtained using the pS2 (TFF1) promoter, an endogenous E2 regulated gene [31] (Figure 2(b)). myrPKCα expression induced a marked increase in basal pS2 promoter activity.
activity and enhanced the stimulatory effect of E2. Treatment with E2 had no effect on the level of myrPKCα expression in Ishikawa cells (not shown).

In HEC-50 endometrial cancer cells, which lack estrogen receptor (ER) [32], activity of the ERE and pS2 promoters was minimal (Figure 3). Expression of active PKCα or treatment with E2 (in the presence or absence of myrPKCα) had no effect on pS2 or ERE promoter activity, indicating that the effects of PKCα and E2 are dependent on ER expression (Figure 3). Accordingly, transfection of HEC-50 cells with pHEGO encoding ERα reconstituted ERE and pS2 transcriptional responses to both E2 and myrPKCα (Figure 4). Expression of ERα in HEC-50 cells also restored the enhancement of E2-stimulated promoter activity by PKCα. (Figure 4). Together, these results (Figures 1–4) indicate that PKCα signaling induces ligand-independent activation of ER-dependent transcription and thereby potentiates responses to E2.

Activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway is one of the most critical steps in endometrial carcinogenesis [11] and has been shown to mediate ligand-independent activation of ER [33, 34]. Moreover, we have previously implicated PKCα in the regulation of Akt in endometrial cancer cells [22]. To investigate the role of PI3K/Akt signaling in PKCα regulation of transcription, we treated Ishikawa cells with pharmacological inhibitors of PI3K (LY29004) or Akt (Akt-I-1/2) [35, 36] and examined their effects on the ERE promoter (Figure 5). Treatment of Ishikawa cells with LY29004 or Akt-I-1/2 significantly inhibited the ability of myrPKCα to enhance activation of the ERE promoter (Figure 5a). Similar results were obtained in HEC-50 cells transfected with ERα (Figure 5b). LY29004 and Akt-I-1/2 treatment resulted in the expected decrease in phosphorylation of Akt and GSK3, respectively, and did not impact expression of myrPKCα (not shown). Thus, the effects of PKCα on E2- and ER-dependent transcription are mediated, in part, by the PI3K/Akt pathway.

To confirm the results, using the ERE and pS2 promoter constructs, we examined expression of a panel of estrogen-dependent genes implicated in endometrial neoplastic transformation [33, 34]. Levels of pS2 (TFF1), lactotransferrin (Ltf), and c-fos mRNA were determined by real-time reverse transcription PCR, in Ishikawa cells stably expressing shRNA to knockdown PKCα. Control cells were transduced with shRNA targeting luciferase [20]. As shown in Figure 6, knockdown of PKCα in Ishikawa cells significantly reduced expression of the estrogen-dependent genes pS2, ltf, and c-fos. PKCα shRNA expressing cells also exhibited the expected decrease in PKCα mRNA levels (Figure 6).

Estrogen is a critical regulator of type I endometrial cancer growth and stimulates proliferation of Ishikawa cells [9, 37–39]. We therefore determined the effect of PKCα knockdown on estrogen-dependent proliferation. E2 treatment stimulated proliferation of Ishikawa cells expressing a control shRNA targeting luciferase, reflected by an increase in the number of viable cells (Figure 7). Knockdown of PKCα significantly reduced the E2-dependent increase in cell number at 72 h and essentially abrogated the E2 proliferative response at 144 h. Cell viability (89%–96%) was not significantly different between cell lines and was not affected by E2 treatment.

Together, these results indicate that PKCα is a critical regulator of ER-dependent gene expression and modulates both E2-stimulated transcription and cell proliferation in ER positive endometrial cancer cells.

4. Discussion

Estrogen, acting through ER, is a major contributor to endometrial proliferation. Indeed, hormone-dependent, type I endometrial cancers are thought to arise due to excess estrogen stimulation, unopposed by progesterone, promoting mitogenesis, atypical hyperplasia, and the transition to malignant adenocarcinoma [4, 8, 11]. In this study, we have shown that activation of PKCα is a critical element of such an estrogenic environment, resulting in estrogen-independent activation of ER-dependent transcription and potentiating the effects of estrogen on both gene expression and endometrial cancer cell proliferation. The primary effect of PKCα is to stimulate basal, unliganded ER transactivation, thereby amplifying estrogen-stimulated promoter activity and enhancing levels of genes linked to endometrial hyperplasia and malignancy.

To confirm the observed interaction of PKCα and ER signaling on estrogen responsive promoters, we examined levels of a subset of estrogen responsive genes (lactotransferrin, pS2/TFF1, and c-fos) implicated in proliferation of normal and transformed endometrial cells and linked to the development of endometrial carcinoma [11, 33, 34, 40, 41]. Knockdown of PKCα in endometrial cancer cells reduced expression of these genes (Figure 6) consistent with their regulation by both ER and PKCα. Accordingly, treatment of breast
Figure 2: PKCα enhances ER-dependent promoter activity. Ishikawa cells were transiently transfected with (a) 0.5 μg pERE-luc or (b) 0.5 μg pPS2-luc and 0.3 μg pCMVβ in the presence or absence of 0.5 μg pmyrPKCα or vector control (pCDNA3). Cells were treated with ±100 nM estradiol (E2), as indicated. Luciferase activity was normalized to β-galactosidase and promoter activity expressed as fold increase over control. Data are mean ± s.e.m of 6 experiments conducted in triplicate.

Figure 3: Estrogen and PKCα responses are ER dependent. HEC-50 cells, lacking ER, were transiently transfected with 0.5 μg pERE-luc or 0.5 μg pPS2-luc and 0.3 μg pCMVβ in the presence or absence of 0.5 μg pmyrPKCα or vector control (pCDNA3). Cells were treated with ±100 nM estradiol (E2), as indicated. Luciferase activity was normalized to β-galactosidase and promoter activity expressed as Relative Light Units (RLU). Data are mean ± s.e.m of 4 experiments conducted in triplicate.

and endometrial cancer cells with phorbol esters, to activate PKC, has been shown to induce expression of pS2 and c-fos and augment their increased levels observed in response to estrogen treatment [41–43].

Cyclin D1 is also an important mediator of estrogen-dependent endometrial cell proliferation and is over expressed in endometrioid tumors [9, 37]. Consistent with interaction of E2 and PKCα mitogenic signaling pathways, we previously demonstrated that PKCα activates the cyclin D1 promoter in endometrial cancer cells [20]. In addition, expression of the cyclin-dependent kinase (CDK) inhibitor p21 is decreased in endometrial cancers, correlating with poorer prognosis [44, 45]. Estrogen-induced Ishikawa cell proliferation paralleled a decline in p21 protein expression [9], whilst progesterone mediated growth inhibition was linked to elevated p21 levels [46]. Expression of p21 was also upregulated in response to knockdown of PKCα [20], suggesting that the CDK inhibitor is a target of both PKCα and estrogen signaling pathways, regulating endometrial cancer cell proliferation.

The PI3K/Akt pathway is commonly dysregulated in type I endometrial cancers. More than 80% of endometrioid carcinomas exhibit loss of the tumor suppressor PTEN and/or activating mutations in PI3K [47–49]. PTEN heterozygous mice develop endometrial hyperplasia and adenocarcinoma, characteristic of human endometrioid tumors [11, 33, 34]. Endometrial tumorigenesis in this model is associated with upregulation of estrogen-stimulated gene expression and ligand-independent activation of ER [34], mediated by Akt [33]. Consistent with these results, we have shown that PKCα
Figure 4: Reconstitution of PKCα regulated, ER-dependent transcription in HEC-50 cells. Cells were transiently transfected with 0.5 μg pHEGO (Erx) and (a) 0.5 μg pEREluc or (b) 0.5 μg pPS2luc and 0.3 μg pCMVβ in the presence or absence of 0.5 μg pmyrPKCα or vector control (pCDNA3). Cells were treated with ±100 nM estradiol (E2), as indicated. Promoter activity was determined as in Figure 2. Data are mean ± s.e.m of 6 experiments conducted in triplicate.

Figure 5: PKCα effects on ER-dependent transcription are mediated by the PI3-kinase/Akt pathway. (a) Ishikawa cells were transiently transfected with 0.5 μg pEREluc and 0.3 μg pCMVβ, in the presence or absence of 0.5 μg pmyrPKCα. (b) HEC-50 cells were transiently transfected with ERα (0.5 μg pHEGO), 0.5 μg pEREluc, and 0.3 μg pCMVβ± 0.5 μg pmyrPKCα or pCDNA3. Cells were treated with ±100 nM estradiol (E2) in the presence or absence of the Akt and PI3K inhibitors, Akt-1/1/2 (1 μM) and LY29004 (10 μM), respectively. Promoter activity was determined as in Figure 2 and expressed as fold increase over the appropriate inhibitor or diluent control. Results are mean ± s.d. (n = 6). *P < 0.05.

is required to maintain Akt activity in endometrial cancer cells [20] and that amplification of estrogen/ER mediated transcription by PKCα is dependent upon the PI3K/Akt pathway (Figure 5).

Phosphorylation of ER has been implicated in regulation of its transcriptional activity and DNA binding [50, 51]. Phosphorylation of serine 167, by Akt, induces activation of ER [33], and phosphorylation of serines 104, 106, and 118
modulates ER interaction with co activators [52]. PKCα-dependent ER phosphorylation and its functional role in endometrial cancer cells remain to be established; however, these latter sites match the consensus substrate sequence for PKC and, since PKCα regulates Akt activity [20], suggest that the effects of PKCα may be mediated by direct or indirect phosphorylation of ER.

5. Conclusions

In summary, we have shown that activation of PKCα induces estrogen-independent activation of ER-dependent gene expression and potentiates the effects of estrogen on transcription. Evidence also implicates PKCα in the regulation of estrogen-dependent endometrial cancer cell proliferation. Thus, PKCα-dependent signal transduction is a critical component of the environment of excessive estrogen and supraphysiologic activation of ER, which is thought to underlie the development of endometrial hyperplasia and endometrioid adenocarcinoma. Furthermore, estrogen exposure may increase PKCα expression and/or activity in endometrial cancer cells [17, 18, 53], providing a potential positive feedback loop to amplify estrogen and ER-dependent responses.

The incidence of endometrial cancer continues to rise, and, despite advances in hormonal and chemotherapy, overall survival has not significantly improved [54–56]. Thus, there is an evident need to develop novel, molecular targeted therapies. PKCα is a critical element in the estrogen, PI3K/Akt, and growth factor/ERK-dependent signal transduction pathways regulating the growth of type I tumors [20–22]. Hence, inhibition of PKCα-dependent signaling would enable the simultaneous targeting of multiple estrogen-dependent and -independent pathways implicated in the development and progression of endometrial carcinogenesis. PKCα specific
inhibitors [57–59] may provide novel avenues, for primary or adjunct therapeutic intervention, to target tumors resistant to current regimens.

**Conflict of Interests**

The authors have no conflict of interests.

**Acknowledgments**

This paper is funded by the Cancer League of Colorado, University of Colorado Cancer Center, and NCI CA125427.

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