Estrogen Suppresses MLK3-Mediated Apoptosis Sensitivity in ER\(^+\) Breast Cancer Cells

Velusamy Rangasamy\(^1\), Rajakishore Mishra\(^1\), Suneet Mehrotra\(^1\), Gautam Sondarva\(^1\), Rajarshi S. Ray\(^1\), Arundhati Rao\(^3\), Malay Chatterjee\(^4\), Basabi Rana\(^2,5\), and Ajay Rana\(^1,5\)

Abstract

Little knowledge exists about the mechanisms by which estrogen can impede chemotherapy-induced cell death of breast cancer cells. 17\(\beta\)-Estradiol (E\(_2\)) hinders cytotoxic drug–induced cell death in estrogen receptor–positive (ER\(^+\)) breast cancer cells. We noted that the activity of the proapoptotic mixed lineage kinase 3 (MLK3) kinase was relatively higher in estrogen receptor–negative (ER\(^-\)) breast tumors, suggesting that E\(_2\) might inhibit MLK3 activity. The kinase activities of MLK3 and its downstream target, c-Jun NH\(_2\)-terminal kinase, were rapidly inhibited by E\(_2\) in ER\(^+\) but not in ER\(^-\) cells. Specific knockdown of AKT1/2 prevented MLK3 inhibition by E\(_2\), indicating that AKT mediated this event. Furthermore, MLK3 inhibition by E\(_2\) involved phosphorylation of MLK3 Ser\(^974\) by AKT, attenuating the proapoptotic function of MLK3. We found that a pan-MLK inhibitor (CEP-11004) limited Taxol-induced cell death and that E\(_2\) accentuated this limitation. Taken together, our findings indicate that E\(_2\) inhibits the proapoptotic function of MLK3 as a mechanism to limit cytotoxic drug–induced death of ER\(^+\) breast cancer cells. Cancer Res; 70(4); 1731–40. ©2010 AACR

Introduction

Estrogens comprise a group of structurally related, hormonally active molecules that regulate cell proliferation, differentiation, and homeostasis (1). Estrogen occurs naturally in several structurally related forms; however, the predominant form that binds strongly to the cognate estrogen receptor α (ER\(_{\alpha}\)) is 17\(\beta\)-estradiol (E\(_2\)). E\(_2\) binds to the nuclear ER\(_{\alpha}\), causing dimerization of receptors that finally drives estrogen action by regulating transcription of target genes (1, 2). It is also reported that E\(_2\) acts through rapid, nongenomic pathways (3, 4), where plasma membrane–localized ER\(_{\alpha}\) or G protein–coupled receptor (GPCR) superfamily, termed GPR30, acts independent of classic ERs to trigger rapid signaling by E\(_2\) (7, 8). Besides ER\(_{\alpha}\), ER\(_{\beta}\) (9–11) has also been reported, whose function is still not well understood in hormonal signaling.

It is well documented that E\(_2\)-mediated pathways play a central role in survival and proliferation of breast cancer cells (1). Therefore, the agents that block either E\(_2\) synthesis or antagonize E\(_2\)-mediated pathways are in clinical use to control the growth of ER\(^+\) breast cancer cells. Thus, the prevailing hypothesis in breast cancer field is that estrogen promotes survival of ER\(^+\) breast cancer cells by inhibiting the proapoptotic cellular machinery. Although, recently, it has been reported that E\(_2\) paradoxically promotes cell death in long-term estrogen-deprived cells (12–15), the detailed mechanism is not well established. The mechanism by which estrogen promotes survival of ER\(^+\) breast cancer cells is also not well understood. It is reported that the rapid action of E\(_2\) activates Src (16–18) and phosphoinositide 3-kinase (PI3K)–AKT (19) pathways, coupled to the insulin-like growth factor-I and epidermal growth factor receptors, which might lead to increased cell survival. Whether E\(_2\) inhibits any proapoptotic kinase to provide growth advantage to E\(_2\)-dependent breast cancer cells is unknown.

Here, we report a role of a proapoptotic kinase, mixed lineage kinase 3 (MLK3), which mediates the rapid action of E\(_2\) on the survival of ER\(^+\) breast cancer cells. MLK3 is a member of MLK subfamily that belongs to the family of mitogen-activated protein kinase kinase kinases (20). The MLK family members are characterized by the presence of signature sequences of serine/threonine and tyrosine kinases within their catalytic domain (20). Previous work by us and others have shown that MLK family members, including MLK3 (20), activate the c-Jun NH\(_2\)-terminal kinase (JNK; refs. 21, 22). We showed that activation of JNK was mediated via direct phosphorylation and activation of upstream kinase, SEK1/MKK4, by MLK3 (21). Furthermore, we reported that MLK3 was directly inhibited by AKT by a direct phosphorylation at a specific residue on MLK3 COOH-terminal regulatory domain (23). Although the detailed function of MLKs, including MLK3, is still unknown, it is reported that MLK3 behaves as a proapoptotic kinase, leading to cell death on trophic factor withdrawal (24) or in response to neurotoxic assault in an...
animal model of Parkinson’s disease (25, 26). The in-depth mechanism by which MLK3 or other MLK family members control survival or death of breast cancer cells is yet to be determined.

In this report, we show that MLK3 kinase activity was significantly higher in ER−, progesterone receptor–negative (PR−) human breast tumors. Interestingly, the kinase activity of MLK3 was inhibited in ER− (MCF7 and ZR75-1) but not in ER+ (SkBr3) cell lines by E2. Furthermore, we show that E2-induced inhibition of MLK3 kinase activity was mediated via PI3K-AKT pathway and more specifically via AKT phosphorylation of MLK3 on Ser674. Induction of cell death mediated via MLK3 in ER+ breast cancer cells was also attenuated by E2. In addition, pharmacologic inhibition of all MLK activity antagonized cytotoxic drug–induced cell death in Er− breast cancer cells, which was completely blocked in the presence of both E2 and pan-MLK inhibitor combination. Taken together, our data show, for the first time, an important role of MLK3 (and possibly other MLKs) in E2-mediated cell death pathways in breast cancer cells.

Materials and Methods

Cell culture and treatments. Human ER+, MCF7 and ZR75-1, and ER−, SkBr3 (American Type Culture Collection), breast cancer cell lines and murine embryonic fibroblasts (provided by Prof. Nissim Hay, University of Illinois, Chicago, IL) were maintained in DMEM containing 10% fetal bovine serum (FBS), 2 mmol/L glutamine, and antibiotics (penicillin G/streptomycin) for 2 d. To elevate the estrogenic effect of exogenously added E2, the cells were cultured in DMEM without phenol red supplemented with 5% charcoal-dextran–stripped FBS, antibiotics, and 2 mmol/L glutamine for further 2 d. For E2 treatment, cells were starved for 12 h in DMEM without phenol red supplemented with 0.2% charcoal-dextran–stripped FBS and treated with or without 10 nmol/L E2 (Sigma) at different time intervals, as indicated. For inhibitor treatment, the starved cells in phenol red–free DMEM were pretreated, wherever indicated, with 50 μmol/L LY294002, 100 nmol/L wortmannin, 20 μmol/L AKT inhibitor VIII, isozyme-selective, AKTi-1/2 (all from Calbiochem), and 10 nmol/L ICI 182,780 (Tocris Bioscience) for 2 to 3 h (as indicated) before E2 treatment. The pan-MLK inhibitor CEP-11004 (kind gift from Cephalon) was dissolved in DMSO and reconstituted in DMEM before treatment. To assess cell death caused by Taxol, cells were pretreated with CEP-11004 (500 nmol/L) for 18 h and subsequently with E2 for 8 h and then with paclitaxel/Taxol (1 μmol/L; Ivax Laboratories) for 24 h.

Immunoblotting. Western blot analysis was performed following protocols described previously (22–24) by incubation with primary antibodies against phospho-AKT, total AKT, poly(ADP-ribose) polymerase (PARP; all from Cell Signaling Technology, Inc.), phospho-JNK (Promega), and total JNK (Santa Cruz Biotechnology). To determine whether MLK3 is the substrate of AKT, phosphoserine/phosphothreonine AKT substrate antibody (Cell Signaling Technology) was used. The specific signals were finally detected using horse-radish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence.

Cell transfection. MCF7 cells were transiently transfected with either Flag-tagged wild-type (WT) MLK3 or Flag-MLK3 S674A mutant using Lipofectamine 2000 (Life Technologies, Inc.) following the manufacturer’s protocol. The endogenous AKT1/2 in MCF7 cells was knocked down as described (27). MCF7 cells were transfected with 100 nmol/L small interfering RNA (siRNA) using Lipofectamine 2000. Forty-eight hours after transfection, cells were treated with E2 for 8 h, as described above.

Cell death assays. Apoptosis of cells was evaluated using a Cell Death Detection ELISAPlus (Roche Diagnostics) kit according to the manufacturer’s instructions. Briefly, the MCF7 cells were transfected with Flag-MLK3 and then treated with E2 (10 nmol/L) for 8 h. The cells were collected by centrifugation at 2000 rpm and analyzed by the Cell Death Detection ELISA kit. Data are expressed as the mean ± SD of three independent experiments.

Immunoprecipitation and kinase assay. The breast tumors and matching normal breast tissue samples were freshly collected and immediately snap frozen in liquid nitrogen following Institutional Review Board–approved protocol. The frozen tissues were homogenized in protein extraction buffer, and endogenous MLK3 (from tissues or cells) was immunoprecipitated with the appropriate antibodies. Endogenous MLK3 was immunoprecipitated by using an antibody raised against the COOH-terminal peptide of MLK3, developed in our laboratory (22–24). Ectopically expressed MLK3 was immunoprecipitated by using antibody against the Flag epitope tag (Sigma). In vitro kinase assay was then performed following our published protocol (22–24). The incorporation of 32P into SEK1 (K-R) was quantified by Phosphoimager (Storm 820, GE Healthcare Bio-Sciences), and the kinase activity was presented as arbitrary phosphoimager units.

Results

MLK3 kinase activity is significantly higher in ER−, PR− breast tumors. To elucidate any role of MLK3 in breast cancer cell apoptosis, we estimated the catalytic activity of MLK3 in primary human breast tumors and matching normal breast tissues. Our results showed that MLK3 kinase activity was significantly higher in some tumors (~10-fold or more) compared with the matching normal breast tissue (Fig. 1). Further analysis was then carried out to determine any link between increased MLK3 activity with the ER and PR status in these breast tumors. Surprisingly, these results revealed that MLK3 kinase activity was ~5-fold higher, exclusively in the ER−, PR− compared with ER+, PR+ tumors (Fig. 1). Additionally, we also measured MLK3 kinase activity in five more tumors. MLK3 kinase activity was about 5- to 12-fold higher in the ER− tumors when compared with the ER+ breast tumors (Supplementary Fig. S1). Taken together, these results suggested the possibility that MLK3 might be negatively regulated by E2-mediated pathways in ER− breast cancer tumors.
Regulation of MLK3 by Estrogen

Estrogen negatively regulates MLK3 and its downstream target JNK in ER+ but not in ER- breast cancer cell lines.

To understand any role of estrogen in regulating MLK3 kinase activity and its downstream events, we treated ER+ MCF7 and ER- SkBr3 cell lines with 10 nmol/L E2 for different time intervals (Fig. 2). The optimal dose of E2 (i.e., 10 nmol/L) to inhibit MLK3 kinase activity was determined by dose course (data not included). Our results showed that MLK3 kinase activity was rapidly inhibited by E2 within 30 minutes of treatment with maximal inhibition (~4.5-fold) after ~8 hours of E2 treatment in the ER+ MCF7 (Fig. 2A) but not in ER- SkBr3 cells (Fig. 2B). To prove that inhibitory action of E2 on MLK3 kinase activity was not cell type dependent, we treated another ER+ breast cancer cell line, ZR75-1, with E2 and estimated MLK3 kinase activity. Endogenous MLK3 kinase activity in ER+ ZR75-1 cells was also inhibited in a similar fashion as in MCF7 cells (Fig. 2C). To confirm that the inhibition of MLK3 kinase activity in ER+ breast cancer cell lines was due to specific action of estrogen, we used the antiestrogen compound ICI 182,780 (28) to block the effect of E2 in MCF7 cell line. The inhibitory effect of E2 on MLK3 kinase activity was completely abrogated by ICI 182,780 compound (Fig. 2D). Our earlier studies showed that MLK3 mediates downstream JNK activation via SEK1/MKK4 (21). Therefore, to determine whether E2-mediated inhibition of MLK3 activity subsequently also leads to inhibition of its downstream JNK kinase activity, we determined the activation status of JNK by using phospho-antibody directed against the activation sites of JNK. Inhibition of MLK3 by E2 also resulted in a corresponding inhibition of JNK activation in ER+ (Supplementary Fig. S2A) but not in ER- (Supplementary Fig. S2B) cell lines. These results showed that E2 indeed inhibits the kinase activities of MLK3 and its downstream JNK in ER+ breast cancer cells.

Estrogen inhibits MLK3 and JNK kinase activities via PI3K-AKT pathway.

Next, we wanted to know the mechanism by which E2 inhibits MLK3 and JNK kinase activities. Our previous results showed that, on incubation with insulin, AKT can phosphorylate and inhibit MLK3 kinase activity (23). Because E2 is reported to activate PI3K-AKT pathway in breast cancer cells (19), it was conceivable that the inhibitory effect of E2 on MLK3 kinase activity might be mediated via PI3K-AKT pathway. To understand the contribution of PI3K-AKT in these events, we first determined whether E2 activates AKT in an ER-dependent manner. Both ER+ MCF7 and ER- SkBr3 cells were treated with E2 for different time intervals, and equal amounts of total cell lysates were Western blotted with phospho-AKT antibody. In the MCF7 cells, E2 was able to activate AKT in a time-dependent manner and the maximal activation was observed in 8 hours of E2 treatment (Fig. 3A), which corresponded with maximal MLK3 inhibition (Fig. 2A). However, E2 was unable to activate AKT in the ER- SkBr3 cells (Fig. 3B), suggesting that E2-mediated activation of AKT involves ER. To examine any role of PI3K-AKT pathway on E2-mediated inhibition of MLK3 activity, the ER+ MCF7 cells were pretreated with two different pharmacologic inhibitors of PI3K-AKT pathway (LY294002 or wortmannin) and then treated with 10 nmol/L E2 for 8 hours. MLK3 kinase assays performed following these treatments showed that E2-induced inhibition of MLK3 kinase activity was antagonized by both the inhibitors of PI3K-AKT pathway (Fig. 3C). These results suggested that E2 inhibits MLK3 kinase activity via activation of PI3K-AKT pathway. We also observed that E2-induced inhibition of JNK (a downstream target of MLK3) was regulated similarly by PI3K-AKT inhibitors (Fig. 3D). Taken together, these results clearly suggest that E2 effects on MLK3 and its downstream target JNK are mediated via PI3K-AKT pathway. However, these results do not prove whether it is PI3K or AKT that ultimately causes E2-induced MLK3 inhibition.

Protein kinase B (AKT) mediates the MLK3 inhibition by estrogen.

To understand whether the inhibitory effect of E2 is mediated directly via AKT in ER+ MCF7 cells, we took advantage of AKT-specific inhibitor, AKT1-1/2, which is reported to inhibit AKT specifically (29). The cells were pretreated with AKT inhibitor followed by treatment with E2. As observed earlier with the PI3K inhibitors, E2 was unable to inhibit MLK3 kinase activity when the cells were pretreated with AKT inhibitor (Fig. 4A), suggesting that AKT does mediate the inhibitory effect of E2 on MLK3 kinase activity. To determine whether the AKT inhibitor used actually inhibits AKT activity, the inhibitor-pretreated cell lysates were also blotted with phospho-AKT antibody, which showed a complete inhibition of AKT phosphorylation following inhibitor pretreatment (Supplementary Fig. S3). Pharmacologic inhibitors of PI3K and AKT have been used extensively to show direct role of PI3K and AKT in cell signaling. However, use of these inhibitors does not rule out their potential nonspecific effects on other pathways. To show specific role of AKT in E2-induced inhibition of MLK3 kinase activity, we first examined the expression of various AKT isoforms in MCF7 cells. We observed that AKT1 and AKT2 are both expressed in MCF7 cell line (Supplementary Fig. S4). To conclusively determine the involvement of AKT, we then knocked down AKT1 and AKT2 in MCF7 cells by using specific siRNA that was reported to knock down both AKT isoforms (27). Subsequently, we examined whether E2 was able to inhibit MLK3 kinase activity in the absence of endogenous AKT1/2. As shown in Fig. 4B, E2 was able to inhibit MLK3 kinase activity in the parental cells but failed to do so in the absence of endogenous AKT1/2 expression in MCF7 cells. As stated before, we have shown earlier that insulin can inhibit MLK3 kinase activity via direct phosphorylation of MLK3 by AKT (23). To know whether AKT phosphorylates MLK3 on E2 treatment, we performed studies using a phospho-AKT substrate antibody that recognizes the phospho-AKT substrates (30). Endogenous MLK3 was immunoprecipitated from cell lysates as described in Fig. 4A, and the immunoprecipitates were blotted with phospho-AKT substrate antibody. These results showed that endogenous MLK3 was phosphorylated on E2 treatment and this phosphorylation was blocked by AKT inhibitor (Fig. 4C), suggesting that AKT does phosphorylate MLK3 on E2 treatment. To further define that the inhibitory effect of E2 on MLK3 was via AKT-mediated phosphorylation, we used an MLK3 mutant, where the AKT phosphorylation site was mutated (23). This site on MLK3 was initially identified as...
the insulin-regulated phosphorylation site by AKT (23). The MCF7 cells were transfected with either Flag-tagged MLK3 WT or MLK3 Ser674 Ala (MLK3 S674A) mutant and then treated either with E2 or vehicle. In vitro kinase assays performed following immunoprecipitation of ectopic MLK3 using the anti-Flag antibody showed that 10 nmol/L E2 was able to inhibit ectopically expressed WT MLK3, reasonably to a lesser extent compared with endogenous MLK3 (Fig. 4D). Interestingly, E2 was unable to inhibit the activity of MLK3 S674A mutant, suggesting that E2-induced AKT does inhibit MLK3 via direct phosphorylation of the Ser674 site. Taken together, these results convincingly prove that the inhibitory effect of E2 in ER+ breast cancer cells is mediated via phosphorylation of MLK3 by AKT.

**Estrogen inhibits the proapoptotic activity of MLK3.** MLK3 has been shown to cause cell death in neuronal and nonneuronal cells (23, 24), whereas E2 in most breast cancer cells, especially in ER+ ones, causes survival (31, 32). E2 has also been shown to have trophic effects on neuronal cells (33–35). Because MLK3 and E2-mediated pathways have opposing effects, we wanted to know whether proapoptotic activity of MLK3 is antagonized by E2 in ER+ breast cancer cells.

To understand the effect of E2 on proapoptotic activity of MLK3, MCF7 cells were transfected with WT MLK3 (which was shown earlier to be constitutively active; ref. 21) and then treated with either E2 or vehicle for 8 hours. The cell lysates were blotted with anti-PARP antibody to determine the effect of E2 on cell death induced by MLK3 expression. We observed that MLK3 overexpression resulted in PARP cleavage, which was significantly attenuated following E2 treatment (Fig. 5A), suggesting that E2 attenuates the cell death activity of MLK3. We also measured the extent of cell death in these cells by using the apoptosome assay. In this assay, E2 again antagonized the cell death function of MLK3 (Fig. 5B). Collectively, these results suggest that E2 downregulates the proapoptotic function of MLK3 in ER+ breast cancer cells.

**MLK3-mediated cell death by cytotoxic drug is attenuated by estrogen.** Cytotoxic drugs, such as Taxol, are generally used to induce cell death in cancer cells, including breast cancer cells (36). Our previous studies have shown that Taxol and other cytotoxic drugs induce MLK3 kinase activity.6 We thus wanted to know whether Taxol-induced cell death in ER+ MCF7 cells is also antagonized by E2, and any possible link of endogenous MLKs in mediating this effect. To perform these studies, we used the available pan-MLK inhibitor CEP-11004 that inhibits the activities of all MLK family members, including MLK3 (25, 26, 37). The cells were pretreated with CEP-11004 for 18 hours followed by treatment with E2 for 8 hours and then with Taxol for 24 hours, as indicated in Fig. 6A. The cell death in these cells was determined by examining the cleavage of PARP. Taxol was able to induce cell death in these cells as indicated by increased PARP cleavage (Fig. 6A, lane 4), which was partially blocked by CEP-11004 (Fig. 6A, lane 6), suggesting the participation of MLK group of kinases. Interestingly, Taxol-induced cell death was significantly reduced by E2 (Fig. 6A, lane 5) and completely blocked by E2 and CEP-11004 combination (Fig. 6A, lane 7). These results suggest that MLK members, including MLK3, mediate breast cancer cell death by Taxol, and this effect of Taxol is down-modulated by inhibitory effect of E2 on MLK3 kinase activity.

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6 G. Sondarva, S. Mehrotra, S. Senthivinayagam, A. Rao, B. Rana, and A. Rana, unpublished data.
Discussion

The overall goal of chemotherapy or radiotherapy for breast cancer treatment is to promote cell death in the tumors so that they can undergo regression and not metastasize to different organs. Most of the modalities currently used for breast cancer treatment result in cell death by modulating various cell death pathways (38). Thus, it is clear that any impediment of the normal cell death pathways might lead to drug resistance and breast cancer progression. It is well documented that the steroidal hormone estrogen promotes cell survival and proliferation of breast cancer cells by regulating the downstream signaling pathways via genomic and non-genomic actions of the hormone (1, 3, 39). Therefore, in ER+ breast cancer cells, agents that antagonize ER signaling pathway or block estrogen synthesis are expected to be effective in promoting cell death (40). Detailed understanding of the pathway by which estrogen promotes cell survival and identification of potential targets are necessary to design rational therapeutic approaches to combat estrogen-dependent breast cancer.

Here, we show that the proapoptotic kinase MLK3 is a target of ER and define the mechanism by which ER antagonizes cell survival and proliferation of breast cancer cells by regulating the downstream signaling pathways via genomic and non-genomic actions of the hormone (1, 3, 39). Therefore, in ER+ breast cancer cells, agents that antagonize ER signaling pathway or block estrogen synthesis are expected to be effective in promoting cell death (40). Detailed understanding of the pathway by which estrogen promotes cell survival and identification of potential targets are necessary to design rational therapeutic approaches to combat estrogen-dependent breast cancer.

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Figure 2. E2 inhibits endogenous MLK3 in ER+ but not in ER− cell lines. A, MCF7 cells were treated with 10 nmol/L E2 at different time points, as indicated. MLK3 was immunoprecipitated and subjected to 

in vitro

kinase assays. Equal expression of MLK3, used for kinase assays, was determined by anti-MLK3 immunoblotting. B, SkBr3 cells were treated with E2 similarly as described in A. MLK3 was immunoprecipitated and subjected to kinase assay as described in Fig. 1. C, ZR75-1 cells were treated with 10 nmol/L E2 at indicated time points. MLK3 kinase activity was measured as described above. D, MCF7 cells were treated with 10 nmol/L ICI 182,780 for 3 h before E2 (10 nmol/L) treatment, and MLK3 kinase activity was measured. These data represent one of three similar experiments (for A and B) and one of two (for C and D).
MLK3 signaling. To define the role of MLK3 in breast cancer cells, we measured MLK3 kinase activities in primary breast tumors as a first step. We were perplexed initially to observe that, in some tumors, the MLK3 kinase activities were very high compared with others and also to the matching normal tissues (Fig. 1; Supplementary Fig. S1). These results suggested that MLK3 might have some yet-to-be-identified role in breast tumors. Further analysis and comparison of the MLK3 kinase activities with the ER and PR status of the breast tumors analyzed showed that there was a direct correlation between ER and PR expression with MLK3 kinase activities in these tumors. Our data clearly showed that MLK3 kinase activities were significantly higher in ER−, PR− tumors (Fig. 1; Supplementary Fig. S1). These results suggested the possibility that estrogen might antagonize MLK3 activity and prompted us to examine the role of estrogen in the regulation of MLK3 kinase activities. The treatment of ER+ breast cancer cells with 10 nmol/L E2 significantly inhibited MLK3 kinase activities within 30 minutes (Fig. 2A); however, MLK3 was maximally inhibited at 8 hours of E2 treatment, which might be due to genomic action of E2 for sustained inhibition. Nonetheless, irrespective of genomic or nongenomic action of E2 on MLK3 kinase activity, our data clearly suggest that the E2 effect was specific because the antiestrogen compound ICI 182,780 blocked the inhibitory action of E2 on MLK3 kinase activity (Fig. 2D).

Activation of JNK in neuronal and nonneuronal cells has been shown to cause cell death (23, 24). It has also been reported that MLK3 causes cell death via JNK activation (41, 42), and thus, one possible downstream effect of E2-mediated MLK3 inhibition in breast cancer cells might be to antagonize MLK3/JNK-induced cell death. We also observed that inhibition of MLK3 in ER− breast cancer cells leads to inhibition of JNK (Supplementary Fig. S2), suggesting that MLK3 regulates JNK activity in these cells. Whether MLK3 causes cell death in breast cancer cells via JNK activation is yet to be determined. It is also reported that the prosurvival kinase AKT is overexpressed (43, 44) in breast tumors and is activated by E2 (45). Because earlier we reported that AKT directly phosphorylates MLK3 and inhibits its activity (23), it

Figure 3. E2 inhibits MLK3 and its downstream target JNK kinase activities via PI3K-AKT pathway. A, MCF7 cells were treated with 10 nmol/L E2 for different time intervals, as indicated, and Western blotting was done using anti–phospho-AKT and anti-AKT antibodies. B, SKBr3 cells were treated with E2 and Western blotting was done as described in A. C, MCF7 cells were pretreated with PI3K inhibitors LY294002 (50 μmol/L) or wortmannin (100 μmol/L) for 2 h and then with E2 (10 nmol/L) for 8 h. MLK3 kinase assay was performed as described in Fig. 1. D, cell lysates from C were immunoblotted for anti–phospho-JNK and anti-JNK antibodies. These data represent one of four similar experiments.
was tempting to determine whether estrogen regulates MLK3 activity via the AKT pathway. It is possible that, during breast cancer pathogenesis, the prosurvival pathways regulated by E2 down-modulate the proapoptotic function of MLK3 via AKT activation. We observed that AKT indeed phosphorylated MLK3 following E2 treatment, which was determined by phospho-AKT substrate antibody (Fig. 4C). This conclusion was further confirmed by using the phospho-deficient MLK3 mutant that lacks the AKT phosphorylation site (Fig. 4D).

As prevailing clinical and nonclinical data support the prosurvival functions of estrogen pathway, it was expected that probably inhibition of MLK3 by E2 will promote survival of breast cancer cells. The inhibition of MLK3 kinase activity by E2 attenuated the proapoptotic functions of MLK3 (Fig. 5), suggesting that, under normal conditions, MLK3 and perhaps other MLKs might regulate the normal cell death pathway in breast epithelial cells. The biggest challenge for any cancer treatment, including breast cancer, is
to overcome the resistance that these cells acquire toward induction of cell death in response to cytotoxic drugs. Based on the facts that (a) MLK3 or other MLK members are proapoptotic kinases and (b) cytotoxic drugs activate MLK3 kinase activity, it is conceivable that inhibition of these protein kinases by E2 might antagonize cytotoxic drug-induced cell death. In fact, pretreatment of ER+ cells with a combination of pan-MLK inhibitor CEP-11004 and E2 completely abrogated cytotoxic drug-induced cell death (Fig. 6A), thus confirming that MLK3 or some yet-to-be-identified MLK family members play an important role in modulating breast cancer cell death.

Based on our current data and published results, we propose a model of MLK3 inhibition by E2 that finally leads to down-modulation of cell death pathway in ER+ breast cancer cells (Fig. 6B). E2 can bind to both ERα and GPCR at the membrane or to the nuclear ERα, resulting in their dimerization. The binding of E2 to membrane-localized ERα or GPCR initiates the nongenomic action of E2 that causes activation of AKT via PI3K. Once AKT is activated, it phosphorylates

Figure 5. Proapoptotic activity of MLK3 is inhibited by E2. A, MCF7 cells were transfected with Flag epitope–tagged MLK3 (WT) and then treated with E2 (10 nmol/L) for 8 h. Cell lysates were immunoblotted with anti-PARP antibody and β-actin was used as a loading control. B, apoptosis was further confirmed in the same extracts using cell death detection ELISA kit (Roche) following the manufacturer’s protocol. These data represent one of three similar experiments.
MLK3 at Ser{sup 674} site, resulting in inhibition of MLK3 kinase activity. Inhibition of MLK3 attenuates JNK activity and finally blocks the transcription of proapoptotic genes via AP-1 transcription factor. At the same time, nuclear ERα binds to estrogen response elements and promotes transcription of prosurvival genes. Therefore, a combination of nongenomic action of E2 on AKT-MLK3-JNK pathway and genomic action via upregulation of prosurvival factors finally shifts the balance toward cell survival.

In conclusion, our data provide an insight toward the role of MLK3 in ER{sup +} breast cancer cell survival. Our biochemical data clearly show that E2 inhibits the MLK3 kinase activities and, by doing so, downregulates its proapoptotic function. This E2 effect is physiologic because MLK3 was highly active in ER{sup +}, PR{sup +} breast tumors but not in ER{sup −}, PR{sup −} tumors. The detail role of MLK3 in ER{sup +}, PR{sup +} breast cancer pathogenesis is a challenging one and is one of our focuses for future studies. Interestingly, our data also show that inhibition of MLK3 by E2 is mediated via AKT phosphorylation of MLK3 on the Ser{sup 674} site. We have also shown earlier that MLK3 is activated by ceramide (22), and it is reported that ceramide inhibits AKT (46). Therefore, it seems likely that agents that can activate MLK3 and inhibit AKT simultaneously might prove beneficial to promote cell death in ER{sup +} breast cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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