Cytosolic phospholipase A2 activation correlates with HER2 overexpression and mediates estrogen-dependent breast cancer cell growth.

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Cytosolic Phospholipase A_2 Activation Correlates with HER2 Overexpression and Mediates Estrogen-Dependent Breast Cancer Cell Growth

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Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) catalyzes the hydrolysis of membrane glycerol-phospholipids to release arachidonic acid as the first step of the eicosanoid signaling pathway. This pathway contributes to proliferation in breast cancer, and numerous studies have demonstrated a crucial role of cyclooxygenase 2 and prostaglandin E_2 release in breast cancer progression. The role of $cPLA_2\alpha$ activation is less clear, and we recently showed that 17β-estradiol (E2) can rapidly activate $cPLA_2\alpha$ in MCF-7 breast cancer cells. Overexpression or gene amplification of HER2 is found in approximately 30% of breast cancer patients and correlates with a poor clinical outcome and resistance to endocrine therapy. This study reports the first evidence for a correlation between $cPLA_2\alpha$ enzymatic activity and overexpression of the HER2 receptor. The activation of $cPLA_2\alpha$ in response to E2 treatment was biphasic with the first phase dependent on *trans*-activation through the matrix metalloproteinase-dependent release of heparin-bound epidermal growth factor. EGFR/HER2 heterodimerization resulted in downstream signaling through the ERK1/2 cascade to promote $cPLA_2\alpha$ phosphorylation at Ser505. There was a correlation between HER2 and $cPLA_2\alpha$ expression in six breast cancer cell lines examined, and inhibition of HER2 activation or expression in the SKBR3 cell line using herceptin or HER2-specific small interfering RNA, respectively, resulted in decreased activation and expression of $cPLA_2\alpha$. Pharmacological blockade of $cPLA_2\alpha$ using a specific antagonist suppressed the growth of both MCF-7 and SKBR3 cells by reducing E2-induced proliferation and by stimulating cellular apoptosis and necrosis. This study highlights $cPLA\alpha_2$ as a potential target for therapeutic intervention in endocrine-dependent and endocrine-independent breast cancer. **(*Molecular Endocrinology* 24: 0000–0000, 2010)**

The phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of the *sn*-2 linkage in membrane glycerol-phospholipids to release arachidonic acid (AA) and lysophospholipid secondary messengers. AA is then converted to bioactive eicosanoid lipid mediators such as prostaglandins, lipoxins, and leukotrienes, which play important regulatory roles in diverse cellular responses. There are three PLA_2 isoform subfamilies: the Ca^{2+}-dependent secretory PLA_2, the Ca^{2+}-independent intracellular PLA_2, and the Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$). The $cPLA_2\alpha$ isoform is constitutively expressed in most cells, and through the modulation of substrate availability, $cPLA_2\alpha$ regulates the rate of AA metabolism to prostaglandins by cyclooxygenases (COX) and so indirectly regulates prostaglandin E_2 (PGE_2) production (1, 2). The AA-based eicosanoid signaling pathway plays an important role in normal cellular homeostasis, inflammation, and pathophysiological conditions. Specifically, eicosanoid signaling has been implicated in the development and progression of malignancy in different tissues including the lung (3), colon (4), prostate (5), and mammary gland (6). Overexpression of AA-metabolizing

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Abbreviations: AA, Arachidonic acid; COX, cyclooxygenase; PLA_2, cytosolic PLA_2; DMSO, dimethylsulfoxide; E2, 17β-estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPR, G protein-coupled receptor; HB, heparin-bound; ICI, ICI 182,780; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; PP2, 4-amino-5-(4chorophenyl)-7-(t-butyl)pyrozolo[3,4-D]pyrimidine; SERM, selective ER modulator; siRNA, small interfering RNA.

enzymes, principally COX-2, can be detected in many breast tumors and correlates with poor patient prognosis (7). COX inhibition decreases cell growth and promotes chemotherapy-induced apoptosis in breast cancer cells (8); epidemiological evidence also links the chronic use of COX-2 inhibitors with a reduced risk of breast cancer development (9, 10).

Recent data have suggested a link between eicosanoid signaling and estrogen-stimulated signaling events in breast cancer cells, at the level of both cPLA$_2\alpha$ and COX-2 activity (11). The eicosanoid pathway has a potential role in estrogen-responsive breast cancer through a positive feedback loop, where COX-2 transcription is upregulated by estrogen through epidermal growth factor receptor (EGFR) *trans*-activation (12), and COX-2 activity stimulates aromatase activity with important consequences for tumor cell proliferation (13). The mitogenicity of circulatory estrogens exerts a critical effect on the etiology and progression of breast cancer, where cumulative exposure of the mammary epithelium to estrogens is a significant risk factor (14, 15). The effects of estrogens, including the most biologically active 17β-estradiol (E2), are driven through the specific estrogen receptors (ERs) α and β (reviewed in Ref. 16). Antagonism of these receptors serves as the basis for therapeutic intervention in breast cancer using selective ER modulators (SERMs) such as tamoxifen and fulvestrant (17–19). ERs act by regulating gene transcription in the nucleus and by modulating the rapid activation of different signaling pathways from the plasma membrane (16). In particular, rapid activation of ERK1/2 MAPK by E2 through ERα and EGFR *trans*-activation has been reported in breast cancer cells (20).

Several studies have linked rapid estrogen-induced signaling to EGFR *trans*-activation: Filardo and Thomas (21) reported the involvement of the G protein-coupled receptor (GPR)-30 in the activation of matrix metalloproteinase (MMP), release of heparin-bound (HB)-EGF, and activation of EGFR in SKBR3 breast carcinoma cells. Razandi *et al.* (22, 23) demonstrated a direct interaction between ER and G proteins and also found that this interaction triggers a Gαq and G$\beta\gamma$-dependent activation of MMPs leading to EGFR *trans*-activation and downstream signaling to ERK and phosphatidylinositol 3-kinase in breast cancer cells. These E2-induced indirect effects can potentiate the mitogenic action of estrogens and are also involved in the development of endocrine resistance by diverting the effects of E2 to alternative growth factor receptor signaling pathways that are insensitive to SERMs (24). The dependency of growth on estrogens can be circumvented by overexpression of EGFR/c-erbB1 and HER2/c-erbB2 (members of the EGFR family of receptor tyrosine kinases that also include c-erbB3 and c-erbB4), which is frequently found in invasive breast cancer and where it correlates with a decreased sensitivity to endocrine therapy and with poor patient prognosis (25).

Clinical, epidemiological, and molecular studies have investigated the role of eicosanoid signaling in breast cancer, focusing mainly on COX-2 and its metabolite PGE$_2$ (11, 26). Animal models have shown that carcinogen-induced mammary tumor formation can be reduced by either treatment with COX inhibitors (27–29) or genetic ablation of *Cox-2* (30). Conversely, COX-2 overexpression in mouse mammary gland increased tumor formation and potentiated angiogenesis (31, 32). As the major prostaglandin produced by COX-2 in breast cancer (33), PGE$_2$ has been shown to play a key role in many aspects of COX-2-induced tumorigenesis. PGE$_2$ levels are elevated in breast cancer (31), and *in vitro* studies have shown that PGE$_2$ can stimulate both the proliferation (34) and migration (35) of mammary epithelial cells. PGE$_2$ can stimulate the expression of growth-promoting genes such as c-*fos* and VEGF (36) and can also increase aromatase activity and consequent estrogen biosynthesis (13), indirectly contributing to cell proliferation.

Despite the body of data available on the role of COX-2 and PGE$_2$ in breast cancer tumorigenesis, the role of cPLA$_2$ in the cross talk between the estrogen and the eicosanoid signaling pathways in estrogen-responsive breast cancer remains unclear. cPLA$_2$ is involved in the rapid estrogen-induced responses in the colon (37) and in embryonic membranes (38). Previous work from our laboratory showed that low concentrations of E2 rapidly promote the activation of cPLA$_2\alpha$ in the MCF-7 breast cancer cell line, impacting on the rapid, estrogen-driven transient rise in intracellular Ca^{2+} concentration. cPLA$_2\alpha$ was activated through ERK1/2 MAPK-dependent phosphorylation on Ser505 and intracellular translocation to perinuclear membranes (39). Here we have identified the receptors and characterized the molecular mechanisms involved in the rapid estrogen-induced activation of cPLA$_2\alpha$ in both endocrine-sensitive and endocrine-resistant breast cancer cells.

Results

E2 rapidly and transiently stimulates cPLA$_2\alpha$ phosphorylation through ER-dependent ERK1/2 activation in MCF-7 cells

We previously showed that E2 stimulated the phosphorylation of cPLA$_2\alpha$ at residue Ser505 within 1 min of treatment in MCF-7 cells (39). To further characterize the the E2-induced cPLA$_2\alpha$ response, we analyzed a time course ranging from 30 sec to 20 min. E2 (10 nM) induced a rapid, transient, and biphasic activation of cPLA$_2\alpha$,

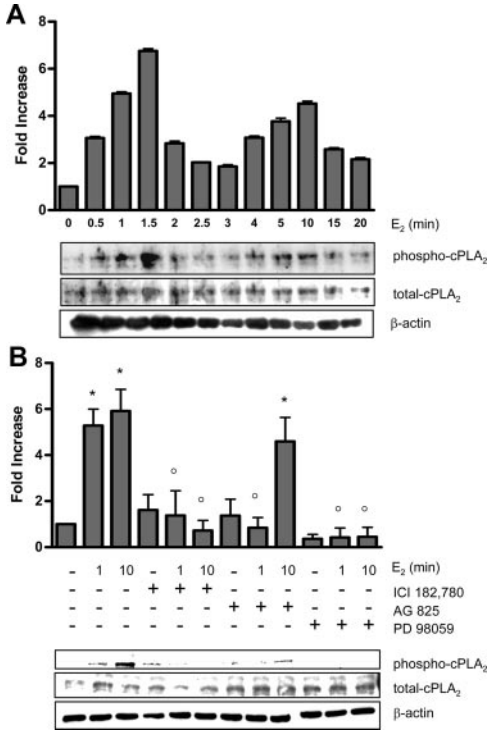

FIG. 1. E2 induces transient phosphorylation of cPLA$_2\alpha$ through ER- and EGFR-dependent MAPK activation. A, Western blot analysis of phospho-cPLA$_2\alpha$ (Ser505) and total cPLA$_2\alpha$ was performed on MCF-7 cells treated with either vehicle (0) or 10 nM E2 at the indicated time points. A representative blot is shown along with densitometric analysis of three independent experiments. B, Western blot analysis of phospho-cPLA$_2\alpha$ (Ser505) and total cPLA$_2\alpha$ was performed on MCF-7 cells treated for 1 and 10 min with either vehicle controls or E2 (10 nM) with or without the inhibitors ICI (10 μM), AG825 (5 μM), or PD98059 (20 μM). β-Actin was used for protein level normalization. Densitometric analysis of three independent experiments is shown with a representative blot. Data are mean values ± SE. *, $P < 0.01$ compared with vehicle-treated control; °, $P < 0.01$ compared with E2-stimulated values at corresponding time points.

with a first peak of phosphorylation starting as early as 30 sec to 2 min after treatment and a second peak detectable from 4–15 min after treatment (Fig. 1A). Time points corresponding to the two maximal peaks of activation (1 and 10 min) were chosen for analysis in all subsequent experiments. The rapid E2-induced activation of signaling pathways is thought to be mediated by an ER localized at or near the plasma membrane. The nature of such a receptor has variously been reported to be either a truncated form of ERα, a lipid-modified form of ERα, or a GPR like GPR30 (40). We previously showed that the rapid activation of cPLA$_2\alpha$ can be induced by both E2 and the membrane-impermeable E2-BSA (39), indicating the involvement of a membrane-localized receptor. Here we show that the specific ER antagonist ICI 182,780 (ICI) blocked the E2-induced phosphorylation of cPLA$_2$ at both 1- and 10-min time points (Fig. 1B).

Phosphorylation of cPLA$_2\alpha$ at Ser505 is mediated by members of the MAPK family (2), and in MCF-7 cells, the rapid effect of E2 on cPLA$_2\alpha$ is specifically driven through ERK1/2 (39). The specific MAPK kinase-1 inhibitor PD98059 blocked the E2-induced phosphorylation of cPLA$_2\alpha$ at 1 and 10 min (Fig. 1B), thus confirming the involvement of ERK1/2 MAPK upstream of cPLA$_2\alpha$. Interestingly, E2 promotes a transient and biphasic phosphorylation of ERK1/2 in MCF-7 cells that mirrors the time-course for cPLA$_2\alpha$ activation (39). Because E2 can activate MAPK through *trans*-activation of EGFR (41, 42), we investigated the role of EGFR in mediating the stimulatory effect of E2 on cPLA$_2\alpha$. Pretreatment of MCF-7 cells with the specific EGFR/HER2 inhibitor AG825 blocked the first rapid peak of E2-induced cPLA$_2\alpha$ phosphorylation but only partially suppressed the subsequent activation at 10 min (Fig. 1B). AG825 is an EGFR kinase inhibitor preferentially selective for HER2 over EGFR, suggesting that HER2 may play a greater role in the earlier phase of cPLA$_2\alpha$ activation.

Rapid E2-induced cPLA$_2\alpha$ activation is dependent on *trans*-activation of EGFR-HER2 heterodimers

Recent evidence demonstrates that in breast cancer cells, E2 promotes EGFR *trans*-activation and downstream signaling through the c-Src-mediated activation of the MMP cascade and the subsequent release of membrane-associated HB-EGF (23, 42). MCF-7 cells were pretreated with the diphtheria toxin mutant CRM197, which inhibits the mitogenic activity of HB-EGF by promoting its internalization from the cell membrane (43). CRM197 blocked E2-induced phosphorylation of cPLA$_2\alpha$ after 1 min treatment but achieved only partial inhibition of E2-induced cPLA$_2$ phosphorylation at 10 min (Fig. 2A). CRM197 also blocked the E2-induced activation of ERK1/2 upstream of cPLA$_2\alpha$ at 1 min, but inhibition was not observed at 10 min. (Fig. 2B). Moreover, pretreating MCF-7 cells with the general MMP inhibitor GM6001 blocked the E2 effect on cPLA$_2\alpha$ phosphorylation at 1 min (Fig. 2C). These data demonstrate that E2-induced cPLA$_2\alpha$ activation at 1 and 10 min is differentially regulated, with the early phase of cPLA$_2\alpha$ activation being dependent on the activation of a MMP cascade at the cell membrane leading to *trans*-activation of EGFR. The later phase of E2-induced cPLA$_2\alpha$ activation at 10 min was largely independent of EGFR *trans*-activation. E2-bound ER binds to and activates the c-Src tyrosine kinase, leading to MAPK activation, through EGFR *trans*-activation or via direct activation of Ras (23, 42, 44). Pretreatment of MCF-7 cells with the specific c-Src inhibitor 4-amino-5-(4chorophenyl)-7-(t-butyl) pyrozolo[3,4-D]pyrimidine (PP2) blocked the E2-induced activation of cPLA$_2\alpha$ at both 1 and 10 min (Fig. 2D), thus confirming the involvement of c-Src in the rapid activation

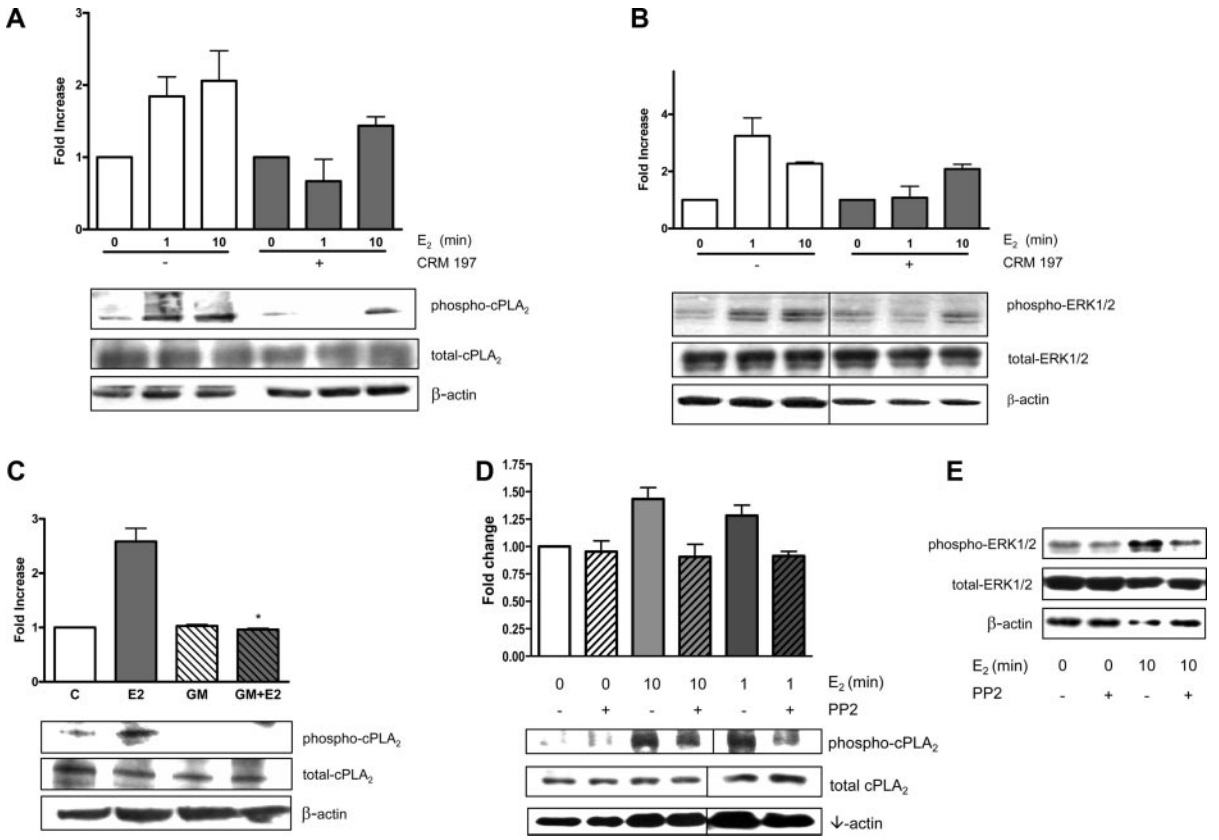

FIG. 2. E2 activates cPLA$_2$ through MMP- and HB-EGF-dependent *trans*-activation of EGFR. A and B, Western blot analysis of cPLA$_2\alpha$ phosphorylation at residue Ser505 (A) and ERK1/2 MAPK phosphorylation at residues Thr202/Tyr204 (B) was performed on MCF-7 cells treated with either vehicle (0) or 10 nM E2, with or without CRM197 (200 ng/ml, 2 h pretreatment) at the indicated time points. A *line* separates noncontiguous lanes on the same gel. C, Phosphorylation of cPLA$_2\alpha$ at residue Ser505 was assessed by immunoblotting in MCF-7 cells treated with either vehicle (control) or 10 nM E2 for 1 min, with or without the MMP inhibitor GM6001 (10 μM). D, Phosphorylation of cPLA$_2\alpha$ at residue Ser505 was assessed by immunoblotting in MCF-7 cells treated with either vehicle (0) or 10 nM E2 for 1 and 10 min, with or without the c-Src antagonist PP2 (100 nM). E, Phosphorylation of ERK1/2 at residues Thr202/Tyr204 was assessed by immunoblotting in MCF-7 cells treated with either vehicle (0) or 10 nM E2 for 10 min, with or without the c-Src antagonist PP2 (100 nM). All blots were stripped and reprobed with total cPLA$_2$ or total ERK1/2 MAPK antibodies. Total cPLA$_2\alpha$ or total ERK1/2 was used for protein level normalization as appropriate. Densitometric analysis of three independent experiments is shown with a representative blot. Data are mean values ± SE. *, $P < 0.01$ compared with E2 stimulation.

of the MMP cascade leading to the EGFR-dependent early phase of cPLA$_2\alpha$ activation at 1 min. Pretreatment of MCF-7 cells with PP2 also blocked the activation of ERK1/2 after 10 min E2 treatment (Fig. 2E). These data suggest that the later phase of cPLA$_2\alpha$ activation at 10 min is largely driven by a c-Src-mediated direct activation of the MAPK cascade that augments the contribution of EGFR *trans*-activation.

EGFR and HER2 can form homodimers and heterodimers with each other and with the other two members of the EGFR family (45). Receptor dimerization and activation leads to *trans*-phosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptors. Activation of EGFR/HER2 heterodimers has been observed in breast cancer cells, resulting in the activation of distinct signaling pathways (46). E2 treatment promoted tyrosine phosphorylation of EGFR in MCF-7 cells within 1 min of treatment (Fig. 3A). The effect was persistent for at least 10 min and abrogated by pretreatment with CRM197, confirming that in this cell line, E2 promotes EGFR *trans*-activation through release of HB-EGF. E2 treatment did not increase phosphorylation of HER2 above basal levels in MCF-7 cells (Fig. 3B), as compared with HCC38 (used here as HER2-negative control) and SKBR3 (HER2-overexpressing positive control). Coimmunoprecipitation studies showed that E2 treatment increased the basal level of association between EGFR and HER2 within a rapid time frame of 1–3 min, corresponding to the first peak of activation of cPLA$_2\alpha$ (Fig. 3C). The effect was also blocked by pretreatment with CRM197. These data suggest that E2 induces a MMP-mediated release of HB-EGF acting in an autocrine fashion to promote *trans*-activation of EGFR through an increase in active EGFR/HER2 heterodimers.

Increased cPLA$_2\alpha$ expression and activity in HER2-overexpressing breast cancer cell lines

To determine whether there was a correlation between EGFR/HER2 heterodimerization and the activation of

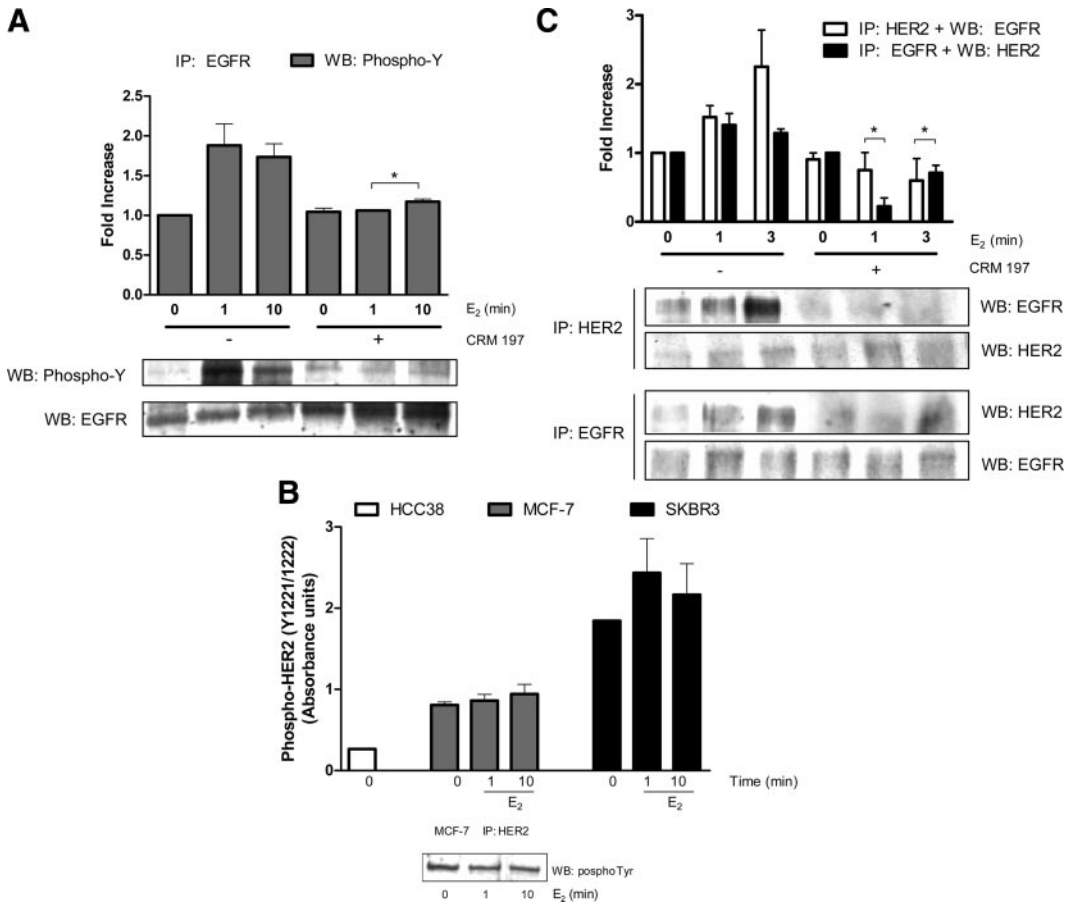

FIG. 3. E2 promotes activation of EGFR-HER2 heterodimers. MCF-7 cells were treated with either vehicle (0) or 10 nM E2 with or without CRM197 (200 ng/ml) at the indicated time points. A and C, Lysates were immunoprecipitated with 2 μg EGFR antibody and blotted for phosphotyrosines (A) or immunoprecipitated with 2 μg of either EGFR or HER2 antibody and blotted for HER2 and EGFR (C). Data are mean values ± SE. *, $P < 0.05$ compared with E2 stimulation without CRM197 at corresponding time points. B, HCC38 (HER2-negative control breast cancer cell line), MCF-7, and SKBR3 cells were treated with either vehicle (0) or 10 nM E2 at indicated time points, and lysates were subjected to ELISA for phospho-HER2 (Tyr 1221/1222). The *lower panel* shows a representative Western blot of MCF-7 lysates immunoprecipitated with 2 μg HER2 antibody and blotted for phosphotyrosines. IP, Immunoprecipitation; WB, Western blot.

cPLA$_2\alpha$, we used the SKBR3 cell line, a breast cancer cell line that is ER negative but HER2 positive and is used as a model for endocrine-resistant, HER2-overexpressing ductal breast carcinoma. Semiquantitative RT-PCR analysis confirmed that SKBR3 cells expressed significantly higher levels of HER2 mRNA compared with MCF-7 cells (53 ± 11% increase, $P < 0.001$). cPLA$_2\alpha$ mRNA levels were also greater (30 ± 4.9%, $P < 0.01$) in SKBR3 cells compared with MCF-7, whereas no significant difference was measured in EGFR (17.5 ± 2.8%) and COX-2 (9.5 ± 1.2%) mRNA levels (Fig. 4A). Western blot analysis confirmed that protein expression levels for EGFR (6.3-fold, $P < 0.001$), HER2 (4.2-fold, $P < 0.01$), and cPLA$_2\alpha$ (12.9-fold, $P < 0.001$) were also greater in SKBR3 cells compared with MCF-7 cells. COX-2 expression was also slightly higher (1.4-fold) but was not statistically significant (Fig. 4B). Quantitative real-time PCR confirmed mRNA expression levels of both HER2 and cPLA$_2\alpha$ were significantly higher in SKBR3 cells when compared with MCF-7 cells (Fig. 4C). To address the question of whether the increased expression of cPLA$_2\alpha$ in SKBR3 cells was coupled to an increased enzymatic activity, we measured hydrolysis of the substrate arachidonoyl thio-phosphatidylcholine *in vitro* (Fig. 4D). SKBR3 cells showed a 2-fold greater cPLA$_2\alpha$ catalytic activity when compared with MCF-7 cells (9.9 ± 0.2 *vs.* 5.8 ± 0.3 nmol/min·ml, respectively), confirming that the greater expression of cPLA$_2\alpha$ in SKBR3 cells translates into a higher enzymatic activity to drive production of AA. To investigate whether the correlation between HER2 and cPLA$_2\alpha$ is a peculiar characteristic of the SKBR3 cell line, we compared a panel of five breast cancer cell lines that differentially expressed HER2 with MCF-7 cells for both HER2 and cPLA$_2\alpha$ mRNA (Fig. 5A) and protein (Fig. 5B) abundance. All cell lines reported to be HER2 overexpressing (BT474, SKBR3, and UACC893) (47) showed higher levels of HER2 expression when compared with MCF-7 cells, at both the mRNA and protein level. SKBR3 and UACC893 also showed higher levels of cPLA$_2\alpha$ mRNA and protein

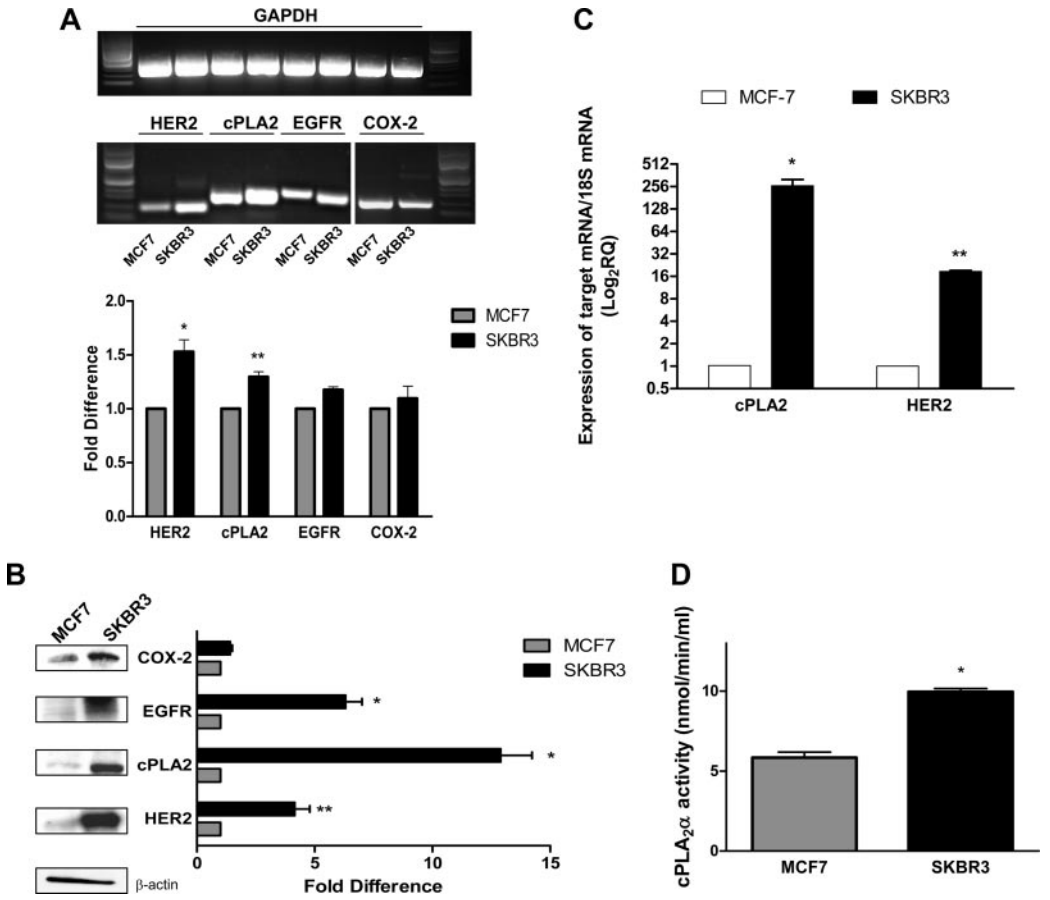

FIG. 4. The endocrine-resistant breast cancer cell line SKBR3 overexpresses EGFR/HER2 and shows increased expression and activity of cPLA$_2$. A and C, Total mRNA was extracted from untreated MCF-7 and SKBR3 cells, reverse transcribed into cDNA, and either subjected to semiquantitative PCR using specific primers for EGFR, HER2, cPLA$_2\alpha$ and COX-2 (expression levels were normalized for GAPDH (a representative agarose gel is shown along with densitometric analysis of six experiments) (A) or subjected to real-time quantitative PCR with specific primers for HER2 and cPLA$_2$ (C). mRNA expression levels were normalized to 18S and expressed as fold difference in relative quantity relative to MCF-7. Data are mean values ± SE. *, $P < 0.001$; **, $P < 0.01$ compared with MCF-7 values. B, Western blot analysis of total EGFR, HER2, cPLA$_2$, and COX-2 was performed on unstimulated MCF-7 and SKBR3 cells. β-Actin was used for protein level normalization. Densitometric analysis of three different experiments is shown with a representative blot. Data are mean values ± SE. *, $P < 0.001$; **, $P < 0.01$ compared with MCF-7 values. D, cPLA$_2$ enzymatic activity was measured in total lysates from MCF-7 and SKBR3 cells. *, $P < 0.01$ compared with MCF-7 values.

compared with MCF-7 cells. BT474 cells had less mRNA but the same amount of cPLA$_2\alpha$ protein as compared with MCF-7 cells. The two cell lines reported to be nonoverexpressing (HCC38 and MDA-MB-231) (47) expressed HER2 mRNA at comparable levels to MCF-7 cells; both of these cell lines also expressed higher levels of cPLA$_2\alpha$ mRNA and protein compared with MCF-7 cells (Fig. 5).

Inhibition of HER2 impacts on cPLA$_2\alpha$ activation in SKBR3 cells

The coupling of HER2 to cPLA$_2\alpha$ activation in SKBR3 cells was investigated by studying the effect of HER2 inhibition on the expression and activation of cPLA$_2\alpha$. To do so, we used both a pharmacological inhibition approach using herceptin and a gene silencing approach using small interfering RNA (siRNA). Herceptin (Trastuzumab) is a recombinant humanized monoclonal antibody directed against the extracellular domain of HER2 that is extensively used in the clinical setting to treat HER2-positive metastatic breast cancer (48). The mechanism of action of herceptin is still not completely clear, but several reports suggest that its action could be explained by an induced impairment of HER2 heterodimerization and consequent blockade of downstream signaling events (48). Treatment of SKBR3 cells with 20 μg/ml herceptin in the presence of serum for 48 h resulted in inhibition of HER2 phosphorylation, with no change in total HER2 protein expression (49). We found that treating cells with herceptin (20 μg/ml) in the presence of E2 (10 nM) resulted in a similar inhibition of HER2 phosphorylation to that found for herceptin in the presence of serum, with no change in total HER2 protein expression levels (Fig. 6A). The reduction in HER2 phosphorylation upon treatment with herceptin in conjunction with E2 was coupled to a reduction in cPLA$_2\alpha$ phosphorylation,

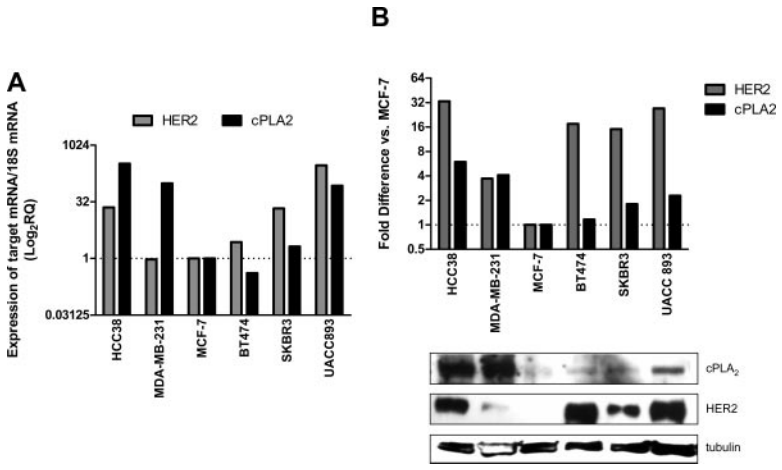

FIG. 5. Analysis of HER2 and $cPLA_2$ expression in a panel of breast cancer cell lines. A, Total mRNA was extracted from untreated HCC38, MDA-MB-231, MCF-7, BT474, SKBR3, and UACC893 cells, reverse-transcribed into cDNA, and subjected to real-time quantitative PCR with specific primers for HER2 and $cPLA_2$. mRNA expression levels were normalized to 18S and expressed as fold difference in relative quantity relative to MCF-7. B, Western blot analysis of total HER2 and $cPLA_2$ was performed on unstimulated HCC38, MDA-MB-231, MCF-7, BT474, SKBR3, and UACC893 cells. α-Tubulin was used for protein level normalization.

with no change in total $cPLA_2\alpha$ protein abundance (Fig. 6A). Treatment with either herceptin alone or E2 alone did not change total expression or the phosphorylation states of either HER2 or $cPLA_2\alpha$. When SKBR3 cells were transfected with a pool of four different siRNA species specific for HER2, expression of the receptor was partially silenced, with protein expression levels reduced to 30% of nontransfected control. Silencing of HER2 reduced $cPLA_2\alpha$ protein expression to 58% of nontransfected control and $cPLA_2\alpha$ phosphorylation levels to 43% of nontransfected control (Fig. 6B), confirming the positive correlation between HER2 overexpression and the abundance of activated $cPLA_2\alpha$ in SKBR3 cells. To rule out any non-sequence-specific effects of gene silencing, a negative nontargeting siRNA control was used that is designed to have at least four mismatches with all known human genes. This negative siRNA had no effect on the expression levels of HER2 or $cPLA_2\alpha$, confirming the specificity of $cPLA_2\alpha$ down-regulation after selective HER2 silencing (Fig. 6B). The HER2 siRNA did not change $cPLA_2\alpha$ expression at the mRNA level as compared with nontransfected or negative siRNA-transfected controls (Fig. 6C), indicating that HER2 exerts a post-translational control of $cPLA_2\alpha$ protein expression.

E2 rapidly promotes $cPLA_2$ phosphorylation in SKBR3 cells through GPR30-dependent EGFR *trans*-activation

In the HER2-positive SKBR3 cells, $cPLA_2\alpha$ was overexpressed and was also constitutively activated (Figs. 4 and 6). However, E2 still promoted $cPLA_2\alpha$ activation above basal levels of phosphorylation, at both 1 and 10 min (Fig. 7A). This effect was blocked by pretreatment with CRM197, demonstrating that the effect of E2 is driven by EGFR *trans*-activation in both ER-positive (MCF-7) and ER-negative (SKBR3) cell lines (Fig. 7A). Unlike in MCF-7 cells, CRM197 treatment fully blocked E2-induced phosphorylation of $cPLA_2\alpha$ in SKBR3 cells at both 1 and 10 min. Pretreatment with the selective EGFR/HER2 inhibitor AG825 also blocked the E2-induced activation of $cPLA_2\alpha$ at 10 min (Fig. 7B). The effect of E2 on ERK1/2 activation showed a different temporal activation profile in SKBR3 to that in MCF-7 cells. In SKBR3 cells, ERK activation started 1 min after E2 stimulation and increased to a maximum at 5 min, remaining constant for at least 10 min, in contrast to the biphasic response observed in MCF-7 cells. CRM197 blocked the stimulation of ERK1/2 activation by E2 in SKBR3 cells over the entire duration of a 10-min time course (Fig. 7D), whereas CRM197 completely blocked only the first transient phase of ERK activation in MCF-7 cells (Fig. 2A). SKBR3 cells are described as ER negative as well as being HER2 positive, but they do express GPR30, which binds E2 to activate MAPK through MMP-mediated EGFR *trans*-activation (42). The rapid effect of E2 on $cPLA_2\alpha$ activation in SKBR3 cells was mimicked by the selective GPR30 agonist G1 and by ICI, which also acts as a GPR30 agonist (21). The effects of E2, G1, and ICI were nonadditive, indicating that E2 and GPR30 agonists may act through a common receptor and signaling pathway in SKBR3 cells (Fig. 7C). The physiological role of GPR30 and its capacity to bind to and mediate the effects of E2 are still controversial.

Researchers have reported the expression of differentially spliced ERα isoforms, namely ERα46 (50) and ERα36 (51), which inhibit the transcriptional activity of wild-type ERα and which could mediate the transduction of estrogen- and antiestrogen-mediated mitogenic signaling from the plasma membrane of endothelial and breast cancer cells (52–54). Western blotting with a specific ERα antibody showed that SKBR3 cells do not express the 66-kDa wild-type ERα, but two bands of approximately 36 and 46 kDa were present that were also detected in MCF-7 cells (Fig. 8A). These bands could represent degradation products, or alternatively spliced receptor isoforms with a similar molecular mass. However, the antibody that was used in this study is directed against an epitope surrounding Ser118 in the A/B domain of ERα, a region that is completely deleted in the ERα36 and ERα46 isoforms (Fig. 8B). Furthermore, when SKBR3 cells were

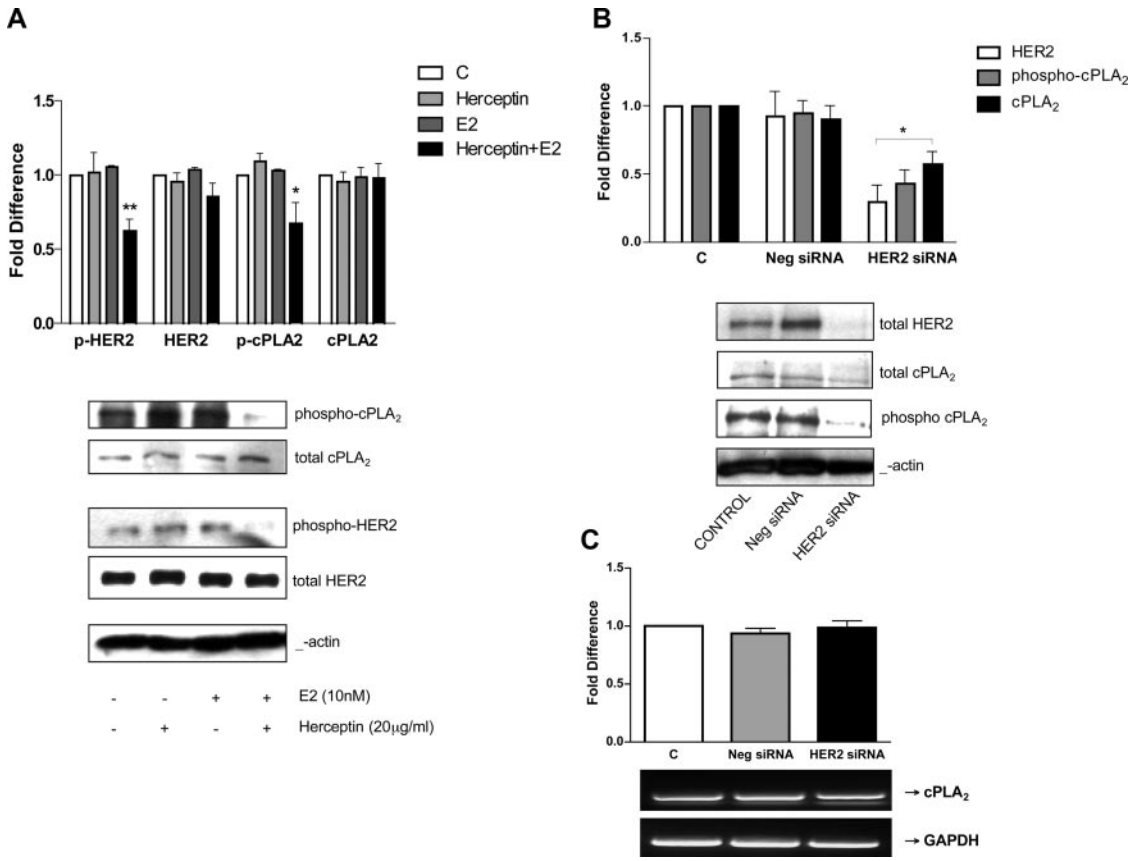

FIG. 6. Inhibition of HER2 in SKBR3 cells decreases cPLA$_2$ activation and expression. Panel A, SKBR3 cells were treated with 20 μg/ml herceptin for 48 h with or without 10 nM E2 as indicated. Western blot analysis of phospho-cPLA$_2\alpha$, total cPLA$_2\alpha$, phospho-HER2, and total HER2 was performed. β-Actin was used for protein level normalization, and densitometric values are expressed as fold difference *vs.* vehicle-treated controls. Data are mean values ± SE. *, $P < 0.01$ compared with control values. Panels B and C, SKBR3 cells were transfected with 100 nM of a pool of four different siRNA targeting HER2. Negative control cells were transfected with 100 nM nontargeting siRNA. At 72 h after transfection, total proteins and mRNA were extracted and subjected to either Western blot analysis for total HER2, phospho-cPLA$_2$ (Ser505) and total cPLA$_2\alpha$, (total cPLA$_2\alpha$ was used for protein level normalization (panel B) (data are mean values ± SE; *, $P < 0.001$ compared with vehicle-treated control) or semiquantitative RT-PCR using specific primers for cPLA$_2$ (panel C) (expression levels were normalized for GAPDH, and a representative agarose gel is shown along with densitometric analysis of two experiments). C, Control.

grown in the presence of E2 for 24 h, the 46-kDa band was not present, and the 36-kDa band was reduced compared with cells grown in the absence of E2 (Fig. 8A). To establish whether the mitogenic effects of E2 (namely, activation of ERK1/2 MAPK and subsequently cPLA$_2\alpha$) in the SKBR3 cells were mediated by GPR30, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to study cell growth (Fig. 8C). Treatment with the antiestrogens ICI and tamoxifen, both of which have been shown to act as GPR30 agonists (21), mimicked the E2-induced increase in cell growth with no additive effect. The selective GPR30 agonist G1 also increased cell growth with no additive effects with E2 (Fig. 8C), suggesting that G1 and E2 were acting through a common receptor to promote cell growth.

Antagonism of cPLA$_2\alpha$ inhibits E2-induced cell proliferation in MCF-7 and SKBR3 cells

The proliferative effect of E2 and EGFR ligands on breast cancer cells is well characterized (16, 25); to investigate whether the eicosanoid signaling pathway and specifically cPLA$_2$ activation is involved in these proliferative events, we measured E2-induced cell growth using the MTT cell growth assay in MCF-7 and SKBR3 cells pretreated with a specific cPLA$_2$ inhibitor (Fig. 9). Treatment of MCF-7 cells with 10 nM E2 resulted in an increased cell growth (24% increase compared with vehicle control). Pharmacological inhibition of cPLA$_2\alpha$ completely abolished the E2-stimulated cell growth. This effect of cPLA$_2$ inhibition on cell growth was comparable to growth inhibition after ICI treatment, which blocked the effect of E2, reducing growth levels (Fig. 9A). In SKBR3 cells, E2 (10 nM) induced a 35% increase in cell growth compared with vehicle-treated control. Pharmacological inhibition of cPLA$_2\alpha$ blocked the cell growth effect of E2 and restored growth levels to control levels. Inhibition of HER2 with herceptin also down-regulated E2-stimulated cell growth to control levels (Fig. 9B). In addition to inhibiting the E2-induced cell growth, the cPLA$_2\alpha$ inhibitor also

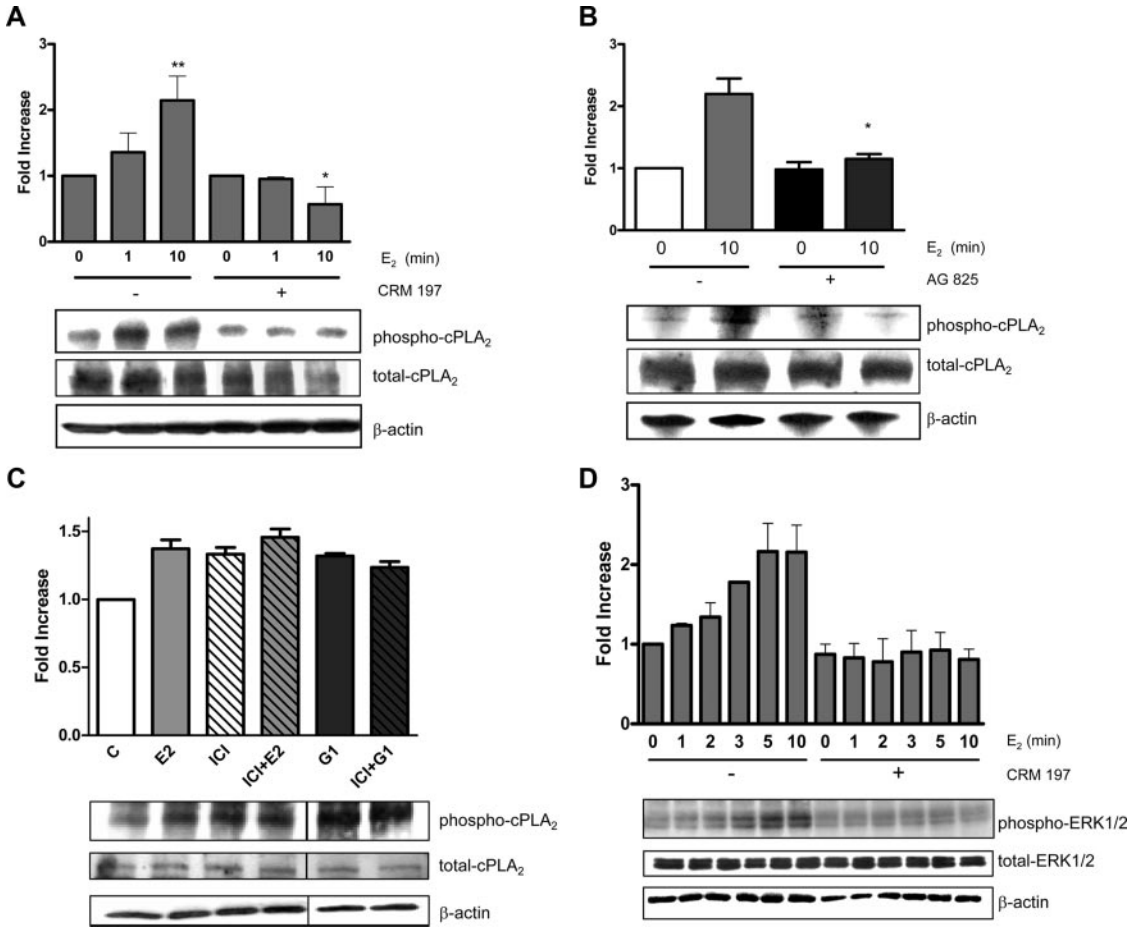

FIG. 7. E2 increases the basal activation of $cPLA_2$ in SKBR3 cells, acting through GPR30-mediated EGFR *trans*-activation. A–C, Western blot analysis of phospho-$cPLA_2\alpha$ (Ser505) and total $cPLA_2$ was performed on SKBR3 cells treated with either vehicle (0) or E2 (10 nM), with or without CRM197 (200 ng/ml) at the indicated time points (A); treated with vehicle (0) or E2 (10 nM), with or without AG825 (5 μM) for 10 min (B); or treated with vehicle (C) or E2 (10 nM) or ICI (10 mM) or the GPR30-selective agonist G1 (100 nM) for 10 min (C). A *line* separates noncontiguous lanes on the same gel. Data are mean values ± SE. **, $P < 0.01$ compared with vehicle-treated control; *, $P < 0.01$ compared with E2-stimulated values at corresponding time point. D, Western blot analysis of phospho-ERK1/2 MAPK (Thr202/Tyr204) and total ERK1/2 MAPK was performed on SKBR3 cells treated with either vehicle (0) or E_2 (10 nM), with or without CRM197 (200 ng/ml) at the indicated time points. Total $cPLA_2\alpha$ or ERK1/2 was used for protein level normalization as appropriate.

reduced MCF-7 cell numbers below control basal levels, which implied a homeostatic role for $cPLA_2\alpha$ in regulating cell viability. Treatment of both MCF-7 and SKBR3 cell lines with the specific $cPLA_2\alpha$ inhibitor increased the incidence of both apoptotic and necrotic cell death compared with vehicle-treated controls (Fig. 9C). In MCF-7 cells, the $cPLA_2\alpha$ inhibitor caused an 85% increase in apoptosis compared with control and a 2.4-fold increase in necrosis. Tamoxifen was used as a positive control, because its effect on cell death in MCF-7 has been previously reported (55). In SKBR3 cells, the inhibition of $cPLA_2$ caused a 2.6-fold increase in apoptosis and a 2.4-fold increase in necrosis, compared with control. Inhibition of HER2 with herceptin, which has been shown to induce cell death (48), also increased cell necrosis and apoptosis in SKBR3 cells, and this response was similar to that observed with the $cPLA_2$ inhibitor (Fig. 9C).

Discussion

The activation of $cPLA_2\alpha$ is the rate-limiting step in the physiological production of AA, which is rapidly metabolized by COX enzymes to produce PGE_2 (1). Prostaglandins regulate many physiological processes through GPR activation leading to the production of second messengers that induce proliferation, migration, apoptosis, and angiogenesis (56). In addition, $cPLA_2\alpha$ can also promote carcinogenesis by liberating membrane lysophospholipids that can induce cell growth through their metabolism to lysophosphatidic acid (57). Consequently, $cPLA_2\alpha$ activity is tightly controlled to maintain low intracellular concentrations of AA in resting cells. However, dysregulation of $cPLA_2\alpha$ activity is detected in many human malignancies, including mammary adenocarcinoma (6). Increased $cPLA_2\alpha$ activity, coupled to increased activity of AA-metabolizing enzymes such as COX-2, leads to high

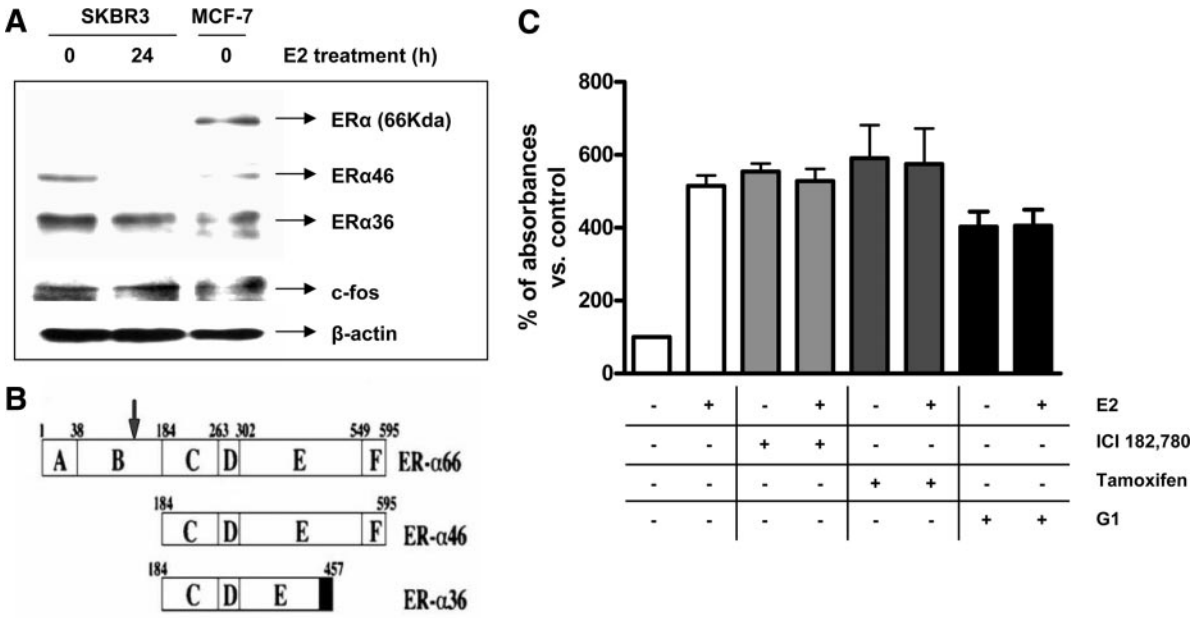

FIG. 8. Involvement of ER isoforms and GPR30 in mediating the effects of E2 in SKBR3 cells. Panel A, Western blot analysis of total ERα was performed on SKBR3 cells treated with either vehicle (0) or E2 (10 nM) for 24 h and on untreated MCF-7 cells. The different isoforms are indicated at their respective molecular weight. Samples were also probed for c-Fos as a positive control for E2 treatment because E2 was reported to up-regulate c-Fos in the SKBR3 cell line (71). β-Actin was used for protein level normalization. Panel B, Structure of the 66-kDa wild-type ERα and the two splice variants ERα36 and ERα46 lacking the N-terminal A/B domain. The different domains (A–F) and amino acid sequence numbers are indicated. An *arrow* indicates the epitope recognized by the ERα antibody used in this study (adapted from Ref. 51). Panel C, MTT cell growth assay was performed on SKBR3 cells treated with either vehicle or E2 (10 nM) with or without ICI (10 μM), tamoxifen (10 μM), or G1 (100 nM) for 48 h. Data are mean values ± SE of three independent experiments.

levels of proliferative eicosanoids (31, 33). Recent studies have focused on the regulatory mechanisms controlling the activity of COX-2 during carcinogenesis. These studies have provided the rationale for the use of nonsteroidal antiinflammatory drugs (such as indomethacin and flurbiprofen) and specific COX inhibitors (such as celecoxib and nimesulide) as chemotherapeutic agents. Despite their efficacy in slowing the progression of malignancy (58), these therapies are often associated with detrimental side effects including gastrointestinal bleeding and cardiovascular toxicity. Other components of the AA-based signaling pathway have been proposed as potential targets for chemoprevention and therapy, including cPLA$_2\alpha$, and therefore, a better understanding of the precise mechanism underlying the activation of cPLA$_2\alpha$ in breast cancer and its role in proliferation would enhance the development of specific pharmacological strategies for the treatment of breast carcinoma and also other malignancies.

We have previously shown that cPLA$_2\alpha$ is expressed in the MCF-7 breast carcinoma cell line and is rapidly activated after treatment with physiological concentrations of E2 (39). In this present study, we investigated the molecular mechanism of E2-induced cPLA$_2\alpha$ activation in breast cancer cell lines that differentially express ERα and HER2. In ERα-positive, HER2-negative MCF-7 cells, E2 elicited a biphasic activation of cPLA$_2\alpha$ that was driven by *trans*-activation of EGFR resulting in activation of the ERK1/2 MAPK cascade. Evidence of a synergism between EGFR and eicosanoid signaling has been described in other experimental systems. EGFR is required for the phosphorylation of cPLA$_2$ induced by neurotensin and EGF in prostate cancer cells (59), and a correlation has been found between COX-2 activity and EGFR activity in breast cancer (12, 60). We found that the initial ERK1/2 activation and downstream phosphorylation of cPLA$_2\alpha$ in response to E2 was dependent on EGFR *trans*-activation in MCF-7 cells, through MMP-dependent release of HB-EGF and the formation of EGFR/HER2 heterodimers. The MMP inhibitor GM6001, the HB-EGF inhibitor CRM197, and the EGFR/HER2 inhibitor AG825 also blocked the phosphorylation of cPLA$_2\alpha$ induced by E2 in this experimental model. E2-induced ERK1/2 activation in breast cancer cells can be mediated by direct interaction of ER with the nonreceptor tyrosine kinase c-Src to activate Ras. E2 also down-regulates MAPK phosphatase 1 (MKP-1) leading to up-regulation of ERK1/2 activity within 10 min of treatment (61). This present study suggests that E2 signals through c-Src-dependent, EGFR *trans*-activation to promote the early phase of ERK1/2 phosphorylation and subsequent cPLA$_2\alpha$ activation within 1 min, whereas the later phase of cPLA$_2\alpha$ activation after 5–10 min is largely driven by EGFR-indepen-

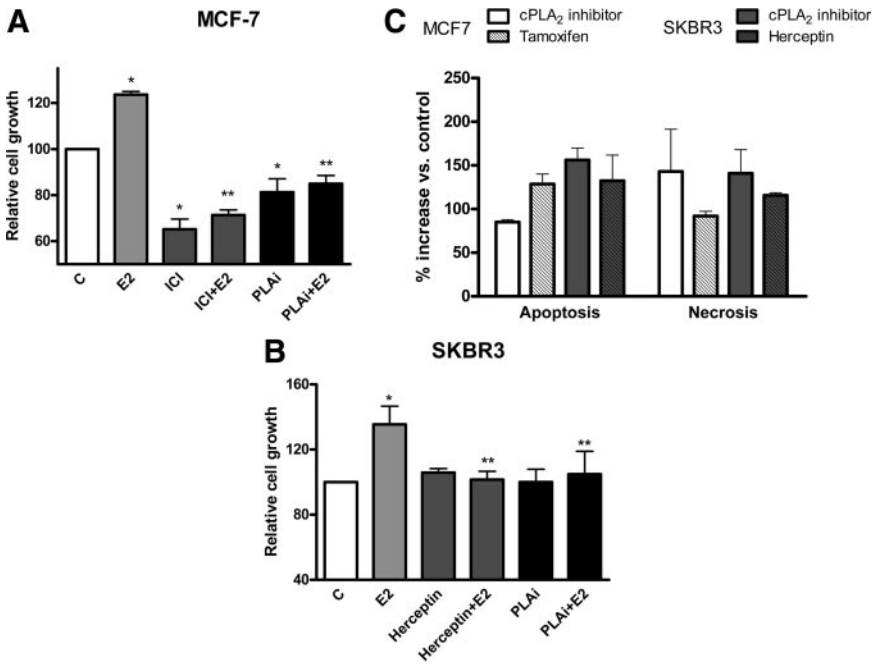

FIG. 9. Pharmacological inhibition of $cPLA_2$ decreases E2-induced cell growth and increases apoptosis and necrosis in both MCF-7 and SKBR3. Panel A, MTT cell growth assay was performed on MCF-7 treated with either vehicle [control (C)] or E2 (10 nM) with or without ICI (10 μM) or the specific $cPLA_2\alpha$ inhibitor (50 nM), and (panel B) on SKBR3 treated with either vehicle (C) or E2 (10 nM) with or without the monoclonal HER2 antibody herceptin (20 mg/ml) or the $cPLA_2\alpha$ inhibitor (50 nM). Data are mean values ± SE. *, $P < 0.01$ compared with vehicle-treated control; **, $P < 0.01$ compared with E2 stimulation. Panel C, An ELISA to detect oligonucleosomes in the cytoplasm or in the cell culture medium was performed on both MCF-7 and SKBR3 treated with the $cPLA_2\alpha$ inhibitor (50 nM) or ICI (10 mM) or herceptin (20 μg/ml) for 48 h. Data are expressed as percent increase of both apoptosis and necrosis compared with vehicle-treated controls.

dent mechanisms through ER-mediated ERK1/2 phosphorylation but which is still c-Src dependent.

Approximately 25–30% of human breast cancers display overexpression or gene amplification of HER2, and its increased expression correlates with poor clinical outcome and with resistance to endocrine therapy (25, 62). EGFR is also overexpressed in 50% of breast tumors and correlates with resistance to hormonal therapy (25). In these tumors, the cross talk between ER and EGFR/HER2 signaling pathways results in a positive feedback cycle of cell survival stimuli. HER2 has the strongest catalytic activity of the four members of the EGFR family, and HER2-containing heterodimers have the greatest capacity for inducing intracellular signaling (46). HER2 is also less sensitive to inactivating signals, and its recruitment into heterodimeric signaling complexes leads to more sustained signaling responses. In the MCF-7 cell line, E2 rapidly promoted an increased dimerization of EGFR with HER2, which was coupled to increased phosphorylation of EGFR but not of HER2. A correlation between overexpression of COX-2 and HER2 gene amplification in breast cancer was previously reported by Ristimäki and colleagues (7). This was subsequently confirmed by the finding that HER2 abundance and activity determines *Cox-2* gene expression (12). This present study is the first report of a correlation between $cPLA_2\alpha$ and HER2 overexpression in a breast cancer cell line. Western blot analysis of phosphorylated $cPLA_2$ showed a constitutive basal activation of $cPLA_2$ in SKBR3 cells that was further increased after E2 treatment. In contrast to MCF-7 cells, the E2-induced activation of $cPLA_2$ in SKBR3 cells was entirely dependent on EGFR *trans*-activation signaling to ERK1/2. SKBR3 cells are ER negative but do express GPR30, which binds to E2 and activates MAPK through MMP-mediated EGFR *trans*-activation (42). The selective GPR30 agonist G1 and ICI both mimicked the effect of E2 and rapidly stimulated the phosphorylation of $cPLA_2\alpha$. Inhibition of EGFR *trans*-activation by CRM197 and AG825 blocked the E2-induced activation of ERK and $cPLA_2$ at all time points analyzed.

If constitutive overexpression of HER2 were the driver for the increased expression and activation of $cPLA_2\alpha$, then inhibition or down-regulation of HER2 would also suppress $cPLA_2\alpha$. Treatment of SKBR3 cells with the anti-HER2 monoclonal antibody herceptin in combination with E2 treatment down-regulated the phosphorylation of both HER2 and $cPLA_2\alpha$, without affecting HER2 or $cPLA_2\alpha$ protein expression levels. Treatment with either herceptin or E2 alone did not elicit any change in the phosphorylation state of either HER2 or $cPLA_2\alpha$, suggesting a synergism between herceptin action and the presence of estrogen. When HER2 protein expression was down-regulated using siRNA, $cPLA_2\alpha$ protein basal expression and phosphorylation were also diminished. This supports the hypothesis that HER2 overexpression drives constitutive $cPLA_2\alpha$ expression and activation in ER-negative breast carcinoma cells. $cPLA_2\alpha$ controls cell proliferation in both normal and malignant thyroid epithelial cells (63, 64), and other reports indicate that $cPLA_2\alpha$ can mediate proliferation in human umbilical vein endothelial cells (65) and also in prostate cancer cells (5). The general PLA_2 inhibitor quinacrine reduced both basal and E2-induced cell growth in MCF-7 cells (66), whereas this present study demonstrates that more specific pharmaco-

logical inhibition of cPLA$_2$ reduced E2-induced cell proliferation of both ER-positive (MCF-7) and ER-negative (SKBR3) breast cancer cells. cPLA$_2$ antagonism in the absence of E2 inhibited MCF-7 but not SKBR3 cell growth. This may reflect the lower basal levels of cPLA$_2\alpha$ activity in the HER2-negative MCF-7 cells, which makes them more sensitive to antagonism of both the homeostatic function of cPLA$_2$ as well as its contribution to E2-induced cell proliferation. For both cell lines, the reduced cell growth was at least in part due to an increase in cell death, because the cPLA$_2$ inhibitor induced both apoptosis and necrosis. The increase in apoptosis could explain why the levels of cell growth in MCF-7 cells fell below the basal level of control when cells are treated with the inhibitor, either alone or in combination with E2.

This study demonstrates a novel role for the rapid, E2-induced *trans*-activation of EGFR/HER2 heterodimers in promoting ERK1/2-induced phosphorylation and activation of cPLA$_2\alpha$ in breast cancer cells that differentially express ER and EGFR/HER2 receptors. HER2 overexpression is a well characterized prognostic marker for invasive breast cancer that is associated with loss of ER expression and resistance to antiestrogen therapy. Our data suggest that HER2 overexpression drives increased cPLA$_2\alpha$ expression and constitutive activation, although loss or inhibition of HER2 can reduce the expression and activation of cPLA$_2\alpha$. In breast cancer cells lacking ER, E2 can increase basal activation of cPLA$_2\alpha$ by *trans*-activating EGFR/HER2, possibly via GPR30. As a consequence, cPLA$_2\alpha$ may contribute to proliferative E2 signaling in tumors that are ER negative and resistant to endocrine therapy. E2 exerts a proliferative effect in breast cancer cells through ER and via EGFR/HER2 in ER-negative tumors. Lipid mediators produced through cPLA$_2\alpha$ activation could play an important role in mediating proliferation of both endocrine-sensitive and endocrine-resistant breast cancer cells. Our data show that pharmacological inhibition of cPLA$_2\alpha$ reduced cell growth *in vitro* through increases in apoptotic and necrotic cell death in both ER-positive and ER-negative cells. Therapeutic strategies to target the eicosanoid signaling pathway have focused mainly on COX-2 inhibition, which results in adverse side effects on the cardiovascular system. This study identifies cPLA$_2\alpha$ as a potential, alternative target for therapeutic intervention in breast cancer.

Materials and Methods

Cell culture

MCF-7, UACC 893, and HCC38 breast carcinoma cells (American Type Culture Collection, Teddington, UK) were routinely grown in Eagle's MEM, Leibovitz L-15, or RPMI 1640 (Sigma-Aldrich, Tallaght, Ireland) culture medium, respectively, supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum (FBS) (GIBCO, Paisley, UK). SKBR3 (American Type Culture Collection), MDA-MB-231, and BT474 breast carcinoma cell lines (Dr. R. J. Santen, University of Virginia School of Medicine, Charlottesville, VA) were maintained in DMEM/F12 (Sigma-Aldrich) supplemented with nonessential amino acids, 2 mM L-glutamine, 100 μg/ml gentamicin, and 10% FBS. All cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37 C with the exception of the UACC 893 cell line, which was maintained at atmospheric CO_2 concentration. For the purpose of experiments, cells were seeded in six-well plates or 10-cm-diameter dishes at 80% confluency and then serum starved for 48 h before treatment at 100% confluency as indicated.

Reagents and antibodies

E2 was purchased from Sigma-Aldrich and dissolved in ethanol before being diluted in cell culture medium to a final concentration of 10 nM. The GPR30-specific agonist G1, the MEK inhibitor PD98059, the c-Src inhibitor PP2, the matrix metalloproteinase inhibitor GM6001, and the specific cPLA$_2\alpha$ inhibitor *N*-c-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl] acrylamide, HCl (67) were obtained from Calbiochem (Nottingham, UK) and dissolved in dimethylsulfoxide (DMSO) (the cPLA$_2\alpha$ inhibitor was dissolved in 75% acetic acid). The HER2/EGFR inhibitor AG825 and the ER inhibitors ICI and tamoxifen were purchased from Tocris (Avonmouth, UK) and dissolved in DMSO or methanol, respectively. The D2189 [Glu^{52}] diphtheria toxin CRM197 was obtained from Sigma-Aldrich and diluted in distilled water to 1 mg/ml. Herceptin (Roche, Clairecastle, Ireland) was diluted in PBS to 10 μg/ml. The bicinchoninic acid protein assay was purchased from Pierce (Northumberland, UK). The Rainbow molecular weight marker, the ECL chemiluminescence reagents, and hyperfilm were from Amersham Bioscience (Little Chalfont, UK). The MTT cell growth assay was from Promega (Southampton, UK). The apoptosis assay was from Roche Applied Science (Burgess Hill, UK). The anti-ERα, anti-cPLA$_2$, anti-HER2, anti-phospho-HER2 (Tyr1221/1222), anti-EGFR, anti-phospho-EGFR (Tyr845), anti-p44/42 MAPK, anti-phospho-p44/42 MAPK (Thr202/Tyr204), the anti-c-Fos, the antimouse IgG horseradish peroxidase conjugate antibodies, and the phospho-HER2 (Tyr1221/1222) ELISA kit were from Cell Signaling Technology (Hitchin, UK). The anti-phospho-cPLA$_2$ antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). The antirabbit IgG horseradish peroxidase conjugate and the anti-β-actin antibodies were from Sigma-Aldrich. All other chemical reagents used were purchased from Sigma-Aldrich, unless otherwise specified.

Immunoprecipitation and Western blotting

Cells were treated with 10 nM E2 or vehicle control for the indicated times. Preincubation with the indicated inhibitors was performed as described. Cells were then transferred onto ice, washed twice with ice-cold PBS, and then ultrasonicated in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, complete mini EDTA-free protease inhibitor mixture tablets (one tablet per 10 ml lysis buffer; Roche) and phosphatase inhibitors]. Samples were clarified by centrifugation at 13,000 rpm for 15 min,

and supernatants were collected and stored at −80 C for subsequent analysis. Total protein concentration was quantified using the bicinchoninic acid assay (68). For immunoprecipitation, equal amounts of soluble cell extracts were incubated with 2 μg of either anti-EGFR or anti-HER2 antibody for 16 h at 4 C with rotation. Washed EZ-view Red Protein A Beads (Sigma-Aldrich) were combined with samples and incubated for 1 h at 4 C on a rotor. Complexes were centrifuged at 13,000 rpm for 3 min, the supernatants were removed, and pelleted beads were washed five times in lysis buffer. Finally, samples were resuspended in 20 μl 2× Laemmli sample buffer (Sigma-Aldrich) and boiled for 5 min at 95 C. For nonimmunoprecipitated samples, total cell extracts were combined with equal amounts of 2× Laemmli sample buffer and heated for 5 min at 95 C.

Solubilized proteins (40 μg) were resolved by SDS-PAGE on 6% (EGFR, HER2, $cPLA_2$, and COX-2) or 10% (ERK1/2) gels (100 V, 90 min). Proteins were then transferred to nitrocellulose membranes (15 V, 45 min to 2 h) with a Trans-Blot SD system (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked in TBS with 0.1% Tween 20 and 5% nonfat dry milk for 1 h at 25 C, incubated with the indicated primary antibody for 16 h at 4 C, and probed with the appropriate secondary antibody for 1 h at 25 C. Membranes were washed three times in TBS with 0.1% Tween 20 at 25 C, and antibody reaction was visualized by enhanced chemiluminescence on an autoradiographic film. Membranes were stripped with Restore Western blot stripping buffer (Pierce Chemical Co., Rockford, IL) for 10 min at 25 C and reprobed with the anti-β-actin or total target protein antibody as indicated to normalize densitometry data for gel loading. Tubulin was used in normalization of the cell line comparison (Fig. 6B) due to large variation in β-actin expression between the cell lines.

RNA isolation and RT-PCR analysis

Total RNA was extracted from the cell lines indicated using the RNeasy mini kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. RNA was finally eluted in diethylpyrocarbonate-treated water (30 μl) and stored at −80 C. The quantity and quality of the extracted RNA was confirmed by absorption measurements at 260 and 280 nm. Single-strand cDNA was synthesized using the ImProm II reverse transcriptase kit (Promega). cDNA was quantified and corrected for loading into RT-PCR mixes. GoTaq polymerase mix (Promega) was used in the PCR amplification. Touchdown PCR was used to amplify cDNA for the indicated number of cycles and annealing temperature range for each primer set used. The RT-PCR product was analyzed on a 1% Tris acetate-EDTA agarose gel and visualized using a UV light source. The abundance of target mRNA detected was normalized in comparison with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control. The sequences for gene-specific forward and reverse primers were designed using the OligoPerfect Designer software program (Invitrogen), unless a different source is specified. Sequences were as follows: for ErbB2/HER2 (GeneID 2064), 5′-CCATAACACCCACCTCTGCT-3′ (forward) and 5′-ACTGGCTGCAGTTGACACAC-3′ (reverse), 20 cycles at 58–68 C; for EGFR (GeneID 1956), 5′-ATGTCCGGGAACACAAAGAC-3′ (forward) and 5′-TTCCGTCATATGGCTTGGAT-3′ (reverse), 40 cycles at 56 C (69); for $cPLA_2\alpha$ (PLA2G4A, GeneID 5321), 5′-ACGTTTCAGAGCTGATGTTT-3′ (forward) and 5′-CTTCCAGCATCTTCATTTTC-3′ (reverse), 30 cycles at 52–62 C; for COX-2 (PTGS2, GeneID 5743), 5′-TGAAACCCACTCCAAACACA-3′ (forward) and 5′-GAGAAGGCTTCCCAGCTTTT-3′ (reverse), 40 cycles at 58–63 C; and for GAPDH (GeneID 2597), 5′-GTCATCATCTCTGCCCCCTCTGC-3′ (forward) and 5′-CGACGGCTGCTTCACCACCTTCT-3′ (reverse), 14 cycles at 52 C (Table 1).

For quantitative real-time PCR, 2 μl cDNA was loaded in a 96-well plate with SYBR Green I Master mix (Roche), and amplification was carried out in a LightCycler 480 (Roche) as follows: a preincubation step at 95 C for 10 min was followed by 45 cycles of denaturation at 95 C for 10 sec, annealing at 60 C for 10 sec, and elongation at 72 C for 10 sec. Efficiencies for each primer set were calculated from the PCR kinetic curve using the linear regression method with LinRegPCR software (70) and used to measure relative quantification of gene expression with the comparative cycle threshold method. All samples were normalized for 18S rRNA expression levels. The sequences for 18S primers were 5′-GTCCCCCAACTTCTTAGAG-3′ (forward) and 5′-CACCTACGGAAACCTTGTTAC-3′ (reverse).

Cell growth and apoptosis assays

Reduction of MTT by mitochondrial respiration was used to measure cell growth. Cells were harvested, counted in a Neubauer chamber, and seeded in a 96-well plate at 10^5 cells per well in medium containing 2% charcoal-stripped FBS. After

TABLE 1. Primer sequences

Gene name	Primer sequences (5′–3′)	Product size (bp)	Number of cycles	Annealing temperature (°C)
ErbB2-HER2				
Forward	CCATAACACCCACCTCTGCT	194	20	58–68
Reverse	ACTGGCTGCAGTTGACACAC			
EGFR (80)				
Forward	ATGTCCGGGAACACAAAGAC	351	40	56
Reverse	TTCCGTCATATGGCTTGGAT			
cPLA2				
Forward	ACGTTTCAGAGCTGATGTTT	352	30	52–62
Reverse	CTTCCAGCATCTTCATTTTC			
COX-2				
Forward	TGAAACCCACTCCAAACACA	187	40	58–63
Reverse	GAGAAGGCTTCCCAGCTTTT			
GAPDH				
Forward	GTCATCATCTCTGCCCCCTCTGC	444	14	52
Reverse	CGACGGCTGCTTCACCACCTTCT			

16 h, cells were stimulated with 10 nM E2 or vehicle control, with or without the indicated inhibitors. Stimulation was repeated after 48 h and carried out for a total 96 h before performing the growth assay, in which cells were incubated with 1 mg/ml MTT for 4 h at 37 C in a humidified atmosphere containing 5% CO_2. The reaction was stopped by the addition of a DMSO solution and solubilization of formazan crystals was allowed for 2 h at 37 C. Absorbance was measured at 570 nm using a Multiskan EX plate reader (Thermo Scientific, Northumberland, UK). A photometric enzyme immunoassay (Roche) was used for the quantitative determination of cytoplasmic histone-associated DNA fragments to measure cell death. Cells were seeded on a 96-well plate at 10^5 cells per well in medium containing vehicle or the indicated inhibitors and incubated for 48 h. Medium was collected and cells lysed for 30 min at 25 C; then both cell lysates and medium supernatants were used for the ELISA following the manufacturer's instructions, and absorbance was measured at 405 nm.

cPLA$_2$ enzymatic activity

MCF-7 and SKBR3 cells were lysed as previously described, and lysates were incubated with 5 μM bromoenol lactone and 200 μM thioetheramide-phosphatidylcholine (Cayman Europe, Tallinn, Estonia) for 15 min at 25 C to inhibit either Ca^{2+}-independent intracellular PLA_2 or Ca^{2+}-dependent secretory PLA_2, respectively. Samples were then incubated with arachidonoyl thio-phosphatidylcholine using a cPLA$_2$ assay kit (Cayman) according to the manufacturer's directions. Briefly, 60 min after incubation, samples were mixed with a solution of 5,5′-dithio-*bis*2-nitrobenzoic acid/EGTA to detect free thiols released by hydrolysis of arachidonoyl thioester bonds by cPLA$_2$. Absorbances were measured at 405 nm using a Multiskan EX iplate reader (Thermo Scientific). Enzymatic activity was calculated using the 5,5′-dithio-*bis*2-nitrobenzoic acid extinction coefficient of 10 mM^{-1}.

RNA silencing

A pool of four different siRNA specific for ErbB2 (NCBI gene ID 2064) was purchased from Dharmacon (Lafayette, CO). Sequences were as follow: siRNA 1, GGACGAAUUCUGCACAAUG; siRNA 2, GACGAAUUCUGCACAAUGG; siRNA 3, CUACAACACAGACACGUUU; and siRNA 4, AGACGAAGCAUACGUGAUG. A nontargeting siRNA with at least four mismatches with all known human genes (Dharmacon D-001210-01) was used as negative control. All siRNAs were resuspended to a 20 μM concentration in a buffer containing 60 mM KCl, 6 mM HEPES (pH 7.5), and 0.2 mM $MgCl_2$. SKBR3 cells were transfected with 100 nM siRNA using DharmaFECT (Dharmacon) and silencing of HER2 expression was assessed by Western blotting over a time course of 24–96 h. Maximal silencing (~65%) was obtained 72 h after transfection; cells were then lysed, and Western blotting for HER2 and cPLA$_2$ was performed as previously described.

Statistical analysis

Densitometric analysis of polyacrylamide and agarose gels was performed using GeneTools software (Syngene, Cambridge, UK). Statistical analysis of the data was performed using paired Student's *t* test for analysis between two groups. One-way ANOVA was used for multiple analyses of more than two groups. P values < 0.05 were considered statistically significant. Data are expressed as mean ± SE of the indicated number of experiments (at least three different experiments performed in duplicate).

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