17β-Estradiol Activates Glucose Uptake via GLUT4 Translocation and PI3K/Akt Signaling Pathway in MCF-7 Cells

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The relationship between estrogen and some types of breast cancer has been clearly established. However, although several studies have demonstrated the relationship between estrogen and glucose uptake via phosphatidylinositol 3-kinase (PI3K)/Akt in other tissues, not too much is known about the possible cross talk between them for development and maintenance of breast cancer. This study was designed to test the rapid effects of 17β-estradiol (E2) or its membrane-impermeable form conjugated with BSA (E2BSA) on glucose uptake in a positive estrogen receptor (ER) breast cancer cell line, through the possible relationship between key components of the PI3K/Akt signaling pathway and acute steroid treatment. MCF-7 human breast cancer cells were cultured in standard conditions. Then 10 nM E2 or E2BSA conjugated were administered before obtaining the cell lysates. To study the glucose uptake, the glucose fluorescent analog 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose was used. We report an ER-dependent activation of some of the key steps of the PI3K/Akt signaling pathway cascade that leads cells to improve some mechanisms that finally increase glucose uptake capacity. Our data suggest that both E2 and E2BSA enhance the entrance of the fluorescent glucose analog 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl-)amino]-2-deoxy-d-glucose, and also activates PI3K/Akt signaling pathway, leading to translocation of glucose transporter 4 to the plasma membrane in an ERα-dependent manner. E2 enhances ER-dependent rapid signaling triggered, partially in the plasma membrane, allowing ERα-positive MCF-7 breast cancer cells to increase glucose uptake, which could be essential to meet the energy demands of the high rate of proliferation. **(*Endocrinology* 154: 1979–1989, 2013)**

Breast cancer is one of the most important diseases worldwide with an annual global fatality of almost 800 000. Approximately 70% of breast cancers diagnosed in women present with elevated levels of estrogen receptor (ER), reflecting their heavy dependence for growth on estradiol. 17β-Estradiol (E2) has important roles in the development of normal breast tissue as well as breast tumors (1, 2). As in other tissues, E2 can affect the function of breast tissue through a variety of integrated and convergent mechanisms (3), that can be classified as follows: 1) ER-independent mechanisms, such as antioxidant effects (4); 2) nonclassical ER-dependent mechanisms such as those mediated by G protein-coupled ER or ER splicing forms (5–7); and 3) classical ER-dependent mechanisms, including nuclear-initiated ER signaling and membrane or cytoplasm-initiated ER signaling. Nuclear-initiated ER signaling by classical ERs is the best-characterized mechanism and involves all transcriptional activities initiated by binding of ERs to specific sequences of the genome called estrogen-responsive elements (8), as well as those initiated by transcriptional factors at other genome sequences, such as activator protein 1 sites (9). Non-nuclear-initiated ER signaling involves classical or alternatively spliced forms of ERs localized either in, or proximal

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Abbreviations: E2, 17β-estradiol; E2BSA, membrane-impermeable form of E2 conjugated with BSA; ER, estrogen receptor; GLUT, glucose transporter; IRS, insulin receptor substrate; 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-*d*-glucose; PI3K, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

to, the plasma membrane. After binding of their cognate physiologic ligand, these receptors can interact with G proteins, signaling cascades, or phosphatases to trigger these nongenomic effects (5, 7, 10, 11). Because ERα does not contain an intrinsic transmembrane domain (12), it requires direct interaction with membrane proteins in order to tether it to the membrane (13). Moreover, palmitoylation of ERα at Cys 447 residue is required for localization of ERα to the plasma membrane and is essential for triggering some of its nongenomic effects (14).

Mammalian cells use glucose as the main substrate for energy production. Glucose is transported into the cells via specialized membrane proteins referred to as glucose transporters (GLUTs) that allow energy-independent transport across the plasma membrane. In non-insulin-dependent tissues such as the mammary gland, GLUT1 is the main GLUT (15), and, moreover, appears to be the predominant GLUT in many tumor types (16, 17). In addition, several groups have demonstrated the presence of other GLUTs, such as GLUT4, in breast cancer cells, and have documented their role in cell cycle progression (18, 19).

ERα signaling has been reported as an enhancer of mitogenic pathways (10) and metabolic mechanisms (20) that involve the insulin-signaling pathway and exert control over insulin secretion (21). These studies demonstrated the importance of E2 in control of the insulin-signaling cascade in traditional insulin-dependent tissue skeletal muscle and adipose tissue (22–25). They also highlighted Akt phosphorylation on its Ser 473 residue, and GLUT4 translocation to the plasma membrane, as key steps in this signaling cascade. Interestingly, however, this functional and mechanistic relationship has not been studied in any other tissue. Tumor cells have a high proliferative rate and dependence on glucose as a metabolic substrate (26) and, in addition, ERα-positive breast cancer cells rely upon E2 for their growth. Accordingly, we hypothesized that, in MCF-7 breast cancer cells, physiologic concentrations of E2 might exert control over some of the key steps of the PI3K/Akt-signaling pathway cascade via nongenomic effects, stimulating glucose metabolism in these cells and affording them an energy advantage.

This study was designed to examine the relationship between the rapid effects of E2, GLUT4 translocation to the plasma membrane, and glucose uptake in MCF-7 breast cancer cells, in order to identify possible novel perspectives on cancer cell metabolism and nutrition.

Materials and Methods

Materials

The following reagents were purchased from Sigma-Aldrich Corp. (St Louis, Missouri): β-estradiol (1,3,5[10]-estratriene-3, 17β-diol) (E2); β-estradiol 6-(O-carboxymethyl) oxime:BSA (E2BSA) contained 30 mol of steroid per mol of BSA; fulvestrant (ICI 182 780). Akt (sc-7126), p85α (sc-71894), GLUT1 (sc-7903), GLUT4 (sc-1606), insulin receptor substrate (IRS)-1 (sc-559) and estrogen receptor α (ERα) (sc-542), Na^+/K^+ ATPase (sc-28800), β-actin (sc-1616) antibodies, as well as protein G-plus agarose for immunoprecipitation and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Phospho-Akt Ser473 was purchased from Cell Signaling Technology (Danvers, Massachusetts; catalog no.4060). Rabbit polyclonal anti-GLUT4 (catalog no. 07–1404; Merck Millipore, Billerica, Massachusetts) (1:200) was purchased for immunocytochemistry purposes. E2BSA was prepared as described by Taguchi et al (27) to remove free E2, freshly in each experiment. 9,10-[^{3}H]palmitic acid (specific activity, 57 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, Massachusetts).

Cell culture

The MCF-7 human breast cancer cells kindly provided by Dr Pedro Sánchez Lazo from Oviedo University, Asturias, Spain were cultured in DMEM (PAA Laboratories, Piscataway, New Jersey) with 10% (vol/vol) fetal calf serum, 2 mM glutamate, 100 IU/mL of penicillin, and 100 μg of streptomycin (Life Technologies, Inc, Gaithersburg, Maryland) at 37°C in a fully humidified 5% CO_2 atmosphere. For experimental purposes and in order to avoid the potential interference of hormones derived from the medium, cells were cultured in phenol red-free DMEM (PAA Laboratories) containing 0.5% fetal calf serum (starvation medium) for 24 hours before the treatment with a 10 nM dose of E2 or of the membrane-impermeant form E2BSA (in this case 10 nM is also the final concentration of E2). ICI 182 780 (10 nM) and LY 294002 (10 μM) were administered, within the 60 minutes before treatment with E2 or E2BSA. These chosen doses are considered as physiologic according to the literature (28–30).

Inhibitors of ERα and PI3K

LY 294002 (Santa Cruz Biotechnology) was used to specifically inhibit PI3K (31, 32), and ICI 182 780 (Sigma-Aldrich Corp) was used as a specific antagonist of ERα (33, 34). To determine the optimal concentrations of both compounds, different doses (10 nM to 100 μM) were tested in cells treated with phenol red-free DMEM for 15, 30, 60, or 120 minutes (data not shown). Additionally, the efficacy of all inhibitors in blocking relevant downstream targets was determined by Western blotting. According to these previous experiments and also to previous literature (29, 35) we decided to treat the cells with these inhibitors for 60 minutes before the hormonal treatment, and with final concentrations of 10 nM for the ICI 182 780 and 10 μM for the LY 294002.

Glucose uptake assay

The fluorescent analog of glucose 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-*d*-glucose (2-NBDG; PeptaNova GmbH, Sandhausen, Germany) was used to measure glucose uptake. Around 5×10^3 cells per well were seeded in 96-well plates and maintained in PRF-complete medium for 24 hours and then maintained in starvation medium for approximately 24 hours until 70–80% confluence. To suppress inter-

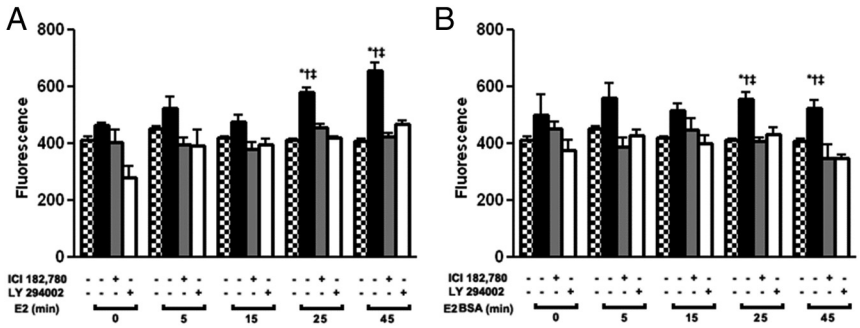

Figure 1. 2-NBDG uptake levels of MCF-7 treated with 0.1% dimethylsulfoxide vehicle ▨, 10 nM E2 ■, 10 nM E2 + 10 nM ICI 182 780 ▩ or 10 nM E2 + 10 μM LY 294002 □ (A); and 0.1% DMSO+BSA vehicle ▨, 10 nM E2BSA ■, 10 nM E2BSA + 10 nM ICI 182 780 ▩ or 10 nM E2BSA + 10 μM LY 294002 □ (B). The histogram shows fluorescence obtained in the different treatment. Values are means ± SEM of 3 independent experiments. Significant differences ($P \leq .05$) are represented with respect to vehicle (*), with respect to combination with ICI 182 780 (†), or with respect to combination with LY 294002 (‡), always in the same time period.

ference of the glucose in the culture medium with fluorescence compound, the medium was removed and changed to Dulbecco's PBS with Ca^{2+} and Mg^{2+} with the corresponding treatment or vehicle, ICI 182 780 or LY 294002. After 60 minutes of treatment, 10 nM E2 or 10 nM E2BSA was added at final concentration, accompanied by a 40 μM dose of the glucose analog 2-NBDG.

After the treatment, the medium was removed and replaced by cold fresh Dulbecco's PBS with Ca^{2+} and Mg^{2+}, and the fluorescence was measured in an FLX-800 micro plate fluorimeter (Bio-Tek Instruments, Inc, Winooski, Vermont) with an excitation wavelength of 485 nm and an emission wavelength of 540 nm (36).

Western blot and immunoprecipitation

After stimulation with E2 or E2BSA and with or without ICI 182 780 or LY 294002, as previously described, MCF-7 cells were harvested and lysed in buffer (50 mM Tris, pH 8; 1% [vol/vol] Triton X-100, 0.1% [vol/vol] sodium dodecyl sulfate [SDS], 0.5% [wt/vol] deoxycholic acid sodium salt, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate) followed by the addition of a protease inhibitor cocktail. To examine GLUT4 translocation to the plasma membrane, we isolated plasma membrane proteins using a commercial plasma membrane protein extraction kit (BioVision, Milpitas, California) and the concentration of protein in the extract was determined with the Bradford reagent (Sigma). A 30-μg sample of protein was subjected to SDS-PAGE (10% polyacrylamide gel) followed by transfer to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia, Barcelona, Spain) as described elsewhere (37). The membranes were blocked by incubation for 1 hour at 4°C with 5% (wt/vol) BSA in Tris-buffered saline (TBS) + 0.1% (vol/vol) Tween-20 (Santa Cruz Biotechnology) to prevent nonspecific protein binding to the nitrocellulose. The membranes were incubated overnight with a 1:1000 dilution of the respective primary antibody at 4°C. After washing with TBS-Tween 20, membranes were incubated for 40 minutes at 4°C with horseradish-peroxidase-coupled secondary antibody in TBS-Tween 20, and the proteins were detected with an enhanced chemiluminescence system.

In order to determine the possible interactions between different molecules, samples of the crude homogenate containing 300 μg of protein were subjected to immunoprecipitation using 2 μg of polyclonal antibodies against ERα and IRS-1. The immune complexes were adsorbed and precipitated using protein G-agarose beads (sc-2002, Santa Cruz) overnight at 4°C on a rocking platform, washed several times with lysis buffer, and then suspended in protein loading buffer (250 mM Tris-HCl, pH 6.8, 8% [wt/vol] SDS, 8 mM EDTA, 35% [vol/vol] glycerol, 2.5% [vol/vol] β-mercaptoethanol, 1% [wt/vol] bromophenol blue) and denatured in boiling water for 5 minutes. The immunoprecipitated proteins were separated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose membranes, and subjected to Western blotting.

Cell labeling with [^{3}H]palmitate and immunoprecipitation

One hour before the hormonal treatment cells were incubated with 0.5 mCi/mL [^{3}H]palmitate at 37°C for 30 minutes. Where indicated, cells were stimulated then with 10 nM of E2 or E2BSA alone or in combination with 10 nM of ICI 182 780. Cells were then washed 3 times in ice-cold PBS and lysed in 100 μL lysis buffer (50 mM Tris, pH 8, 1% [vol/vol] Triton X-100, 0.1% [vol/vol] SDS, 0.5% [wt/vol] deoxycholic acid sodium salt, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1 mM sodium orthovanadate). Then lysates were centrifuged before immunoprecipitation as described previously. Briefly, equal amounts of protein were incubated with 2 μL of anti-ERα antibody and 25 μL of protein G-agarose beads (sc-2002, Santa Cruz) for 3 hours at 4°C. After centrifugation (12 000 x *g* 5 min) and three washes with PBS 1% Tween 20, the radioactivity present in the immunoprecipitated proteins was monitored by counting with a Wallac 1409 Liquid Scintillation Counter.

Immunocytochemistry

Cells were grown on coverslips, and after hormonal treatment for 25 minutes, were fixed on 4% formaldehyde; after blocking with 15% of goat serum, cells were incubated for 2 hours at room temperature with rabbit polyclonal antibody against GLUT4 (catalog no. 07-1404 Merck Millipore). For fluorescent detection, cells were incubated for 1 hour at room temperature with Alexa 488 conjugated goat antirabbit (Life Technologies). Optical sections were acquired using a Leica TCS-SP2-AOBS confocal microscope (Leica, Inc., Deerfield, Illinois) 63× objective PL APO 63X/1.40 Oil Lbd BL at a resolution of 1024 × 1024, without zoom magnification.

Statistical analyses

All data are represented as the mean ± SEM. Differences with $P < .05$ determined by a one-way ANOVA and Tukey post hoc tests were considered statistically significant.

Results

Effect of E2 on 2-NBDG uptake

A fluorescent analog of glucose, 2-NBDG, was used to determine the effect of short-term E2 treatment on glucose

uptake. As shown in Figure 1 both E2 and E2BSA show an increased uptake of the glucose analog, and both seem to do it in a significant way after 25 minutes from the beginning of the experiment ($P < .05$). Additionally this response seemed to be totally blocked to baseline levels by cotreatment with ICI 182 780, and also with LY 294002, which suggests a key role of both ERα and PI3K.

Plasma membrane GLUT-4 levels

To further investigate the possible relationship between short-term treatment with E2 and increased glucose uptake, we checked whether the treatment modulated the GLUT4 translocation via PI3K/Akt signaling pathway. The first step was to determine whether hormonal treatments had any influence on the presence of GLUT4 in the plasma membrane (Figure 2). Although we could observe a small basal fraction of GLUT4 in the plasma membrane, we found that both E2 and E2BSA were able to increase the presence of this insulin-dependent GLUT, already at 5 minutes of treatment ($P < .05$). Further, the presence of GLUT4 in the plasma membrane was limited to baseline levels by treatment with 10 nM ICI 182 780, and 10 μM LY 294002 negated the effect of both E2 and E2BSA almost entirely. As shown in Figure 2, our treatments didn't change the total amount of GLUT4.

Immunofluorescence technique seems to support this finding (Figure 2C). At least with 25 minutes of hormonal stimulation, a specific GLUT4 staining seems to be concentrated in the periphery of the cell, near the plasma membrane, compared with the basal situation.

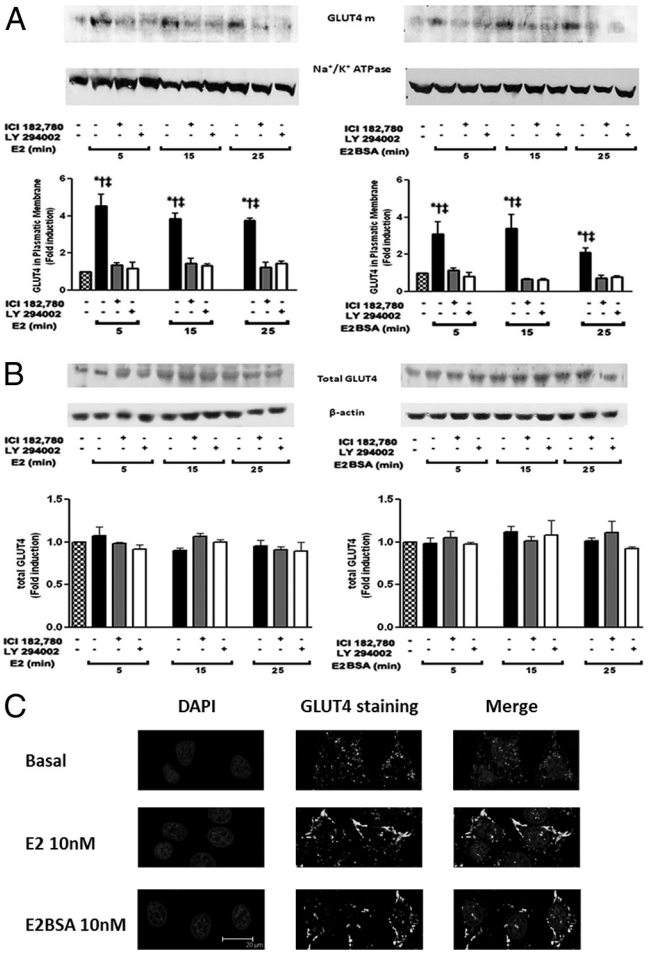

Figure 2. Changes in GLUT4 Plasma Membrane and Total Amount of GLUT4 (panels A and B, respectively) levels of 0.1% Vehicle (−E2/E2BSA), 10 nM E2 , or 10 nM E2BSA, Alone or in Combination with 10 nM ICI 182 780 or 10 μM LY 294002, That Were Both Administered 60 Minutes before the Hormonal Treatment The histogram shows the densitometry analysis of the Western blots. Values are means ± SEM of 3 independent experiments, and they are normalized with respect to the control value (vehicle). Significant differences ($P \leq .05$) are represented with respect to vehicle (*), with respect to combination with ICI 182 780 (†), or with respect to combination with LY 294002 (‡). C, Immunofluorescence of GLUT4 in basal and E2/E2BSA-stimulated cells. Immunofluorescence was performed with anti-GLUT4 rabbit polyclonal antibody and Alexa 488-conjugated IgG. DAPI, 4′,6-diamidino-2-phenylindole.

No changes were observed in the plasma membrane GLUT-1 levels

As proposed in the introduction, GLUT1 is the main GLUT in the mammary gland, providing the cell the basal levels of glucose to satisfy their energy demands. In order to clarify if it had any influence in the increasing glucose uptake we saw, we have analyzed the influence of our treatment on the total amount of this GLUT and over its location on the plasma membrane. As shown in Figure 3, there were changes neither on GLUT1 presence in the plasma membrane nor in the total levels due to our treatment.

Plasma membrane ERα levels

Next we tried to verify the presence of the ERα on the plasma membrane and the effect of the different treatments on its translocation to the plasma membrane. In both cases (E2 and E2BSA) we observed two bands corresponding to two splicing isoforms previously described: ERα66 and ERα46 (Figure 4).

Surprisingly the ERα46 isoform ($P < .05$), but not the ERα66, seems to be mobilized to the plasma membrane responding to the E2 or E2BSA treatment. This effect seems to be ERα-dependent, because it is reversed to baseline levels by ICI 182 780 cotreatment. Meanwhile, LY 294002 also reverses the effect over the presence of ERα46 in the plasma membrane, but this effect remains unexplained and will need extra experiments to reach a complete clarification.

ERα palmitoylation levels

The palmitoylation of the ERα is needed for its approximation to the plasma membrane. To assess the effect of the E2 or E2BSA treatment over the palmitoylation of

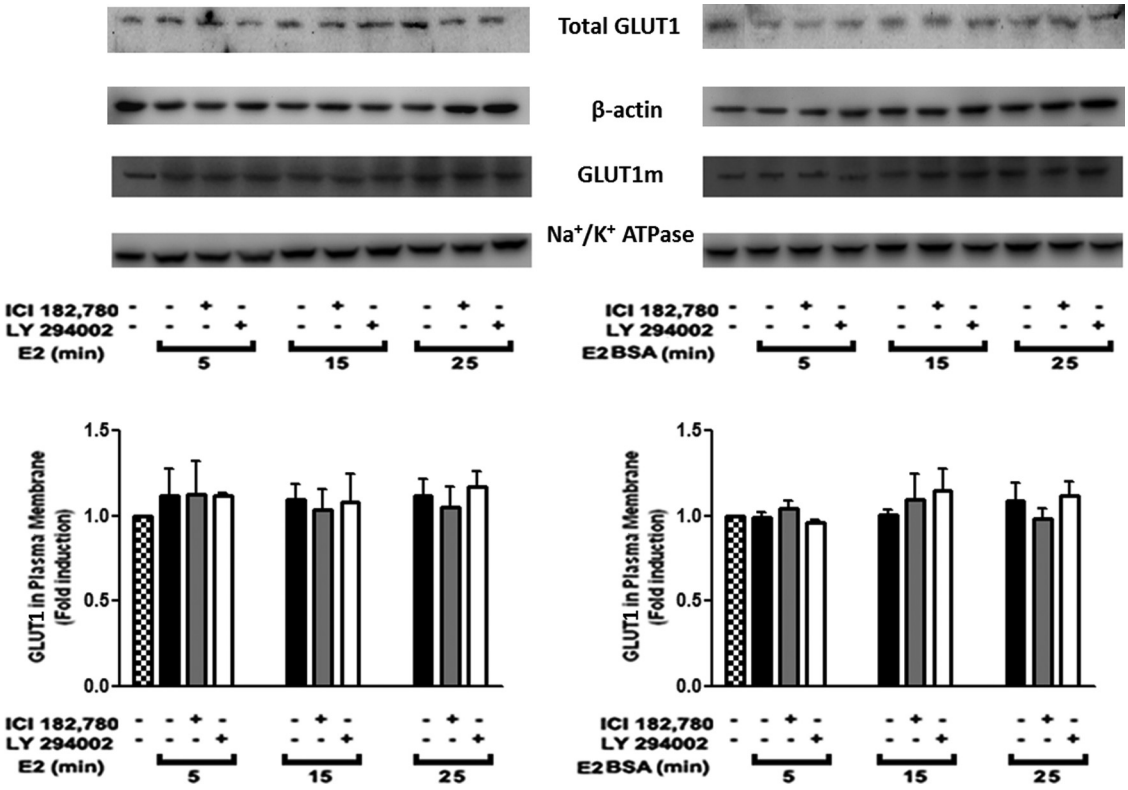

Figure 3. GLUT1 Plasma Membrane Levels of 0.1% Vehicle (-E2/E2BSA), 10 nM E2, or 10 nM E2BSA, Alone or in Combination with 10 nM ICI 12 780 or 10 μM LY 294002, That Were Both Administered 60 Minutes before the Hormonal Treatment The histogram shows the densitometry analysis of the Western blots. Values are means ± SEM of 3 independent experiments and they are normalized with respect to the control value (vehicle). Significant differences ($P \leq .05$) are represented with respect to vehicle (*), with respect to combination with ICI 182 780 (†), or with respect to combination with LY 294002 (‡).

ERα, MCF-7 cells were incubated with 0,5 mCi/mL of [^{3}H]palmitate in the presence of 10 nM dose of E2 or E2BSA alone or in combination with ICI 182 780. In the presence of hormones the amount of [^{3}H]palmitate incorporated to the ERα decreased until the end of the experiment (Figure 5A). This effect was totally reversed in the combination with 10 nM of ICI 182 780 (Figure 5C). It is important to note that although both hormonal treatments decreased this posttranslational modification, the total amount of the ERα did not change during all the course of the experiment (Figure 5B).

E2 and E2BSA induce AKT Ser473 phosphorylation in MCF-7 cells

In accord with the results obtained in the presence of GLUT4 in the plasma membrane, and continuing upstream in the signaling cascade, we next examined Akt ser473 phosphorylation as the previous step to GLUT4 translocation by E2 and E2BSA in MCF-7 cells. We could observe something similar to GLUT4 presence in the plasma membrane; both treatments, E2 and E2BSA, significantly increased Ser473 phosphorylation of Akt (Figure 6), since the first steps of the experiment ($P < .05$). As we observed in earlier experiments, this effect was totally blocked by ICI 182 780. The phosphorylation is inhibited also by the PI3K inhibitor LY 294002, suggesting that this activation is PI3K dependent.

E2 and E2BSA, induce p85α-ERα interaction but not p85α-IRS-1 in MCF-7 cells

We have evaluated the interaction between the regulatory subunit of PI3K (p85α), and IRS-1 as the key step before Akt Ser473 phosphorylation (38). To quantify this interaction we have immunoprecipitated IRS-1, and then we blotted against p85α. We observed that neither E2 nor E2BSA exerted any interaction between p85α and IRS-1 (data not shown).

As an alternative explanation to Akt Ser473 phosphorylation induced by treatment with E2 or E2BSA, we checked the interaction between p85α and ERα (39, 40). For this purpose, we used p85α immunoprecipitation and Western blotting against ERα. We found (Figure 7) that treatment with E2 or E2BSA significantly favored the interaction of both isoforms, ERα66 and ERα46, with p85α ($P < .05$). Again, we observed that cotreatment with ICI 182 780 negated the stimulatory effect of both E2 and E2BSA. Cotreatment with LY 294002 and E2 or E2BSA

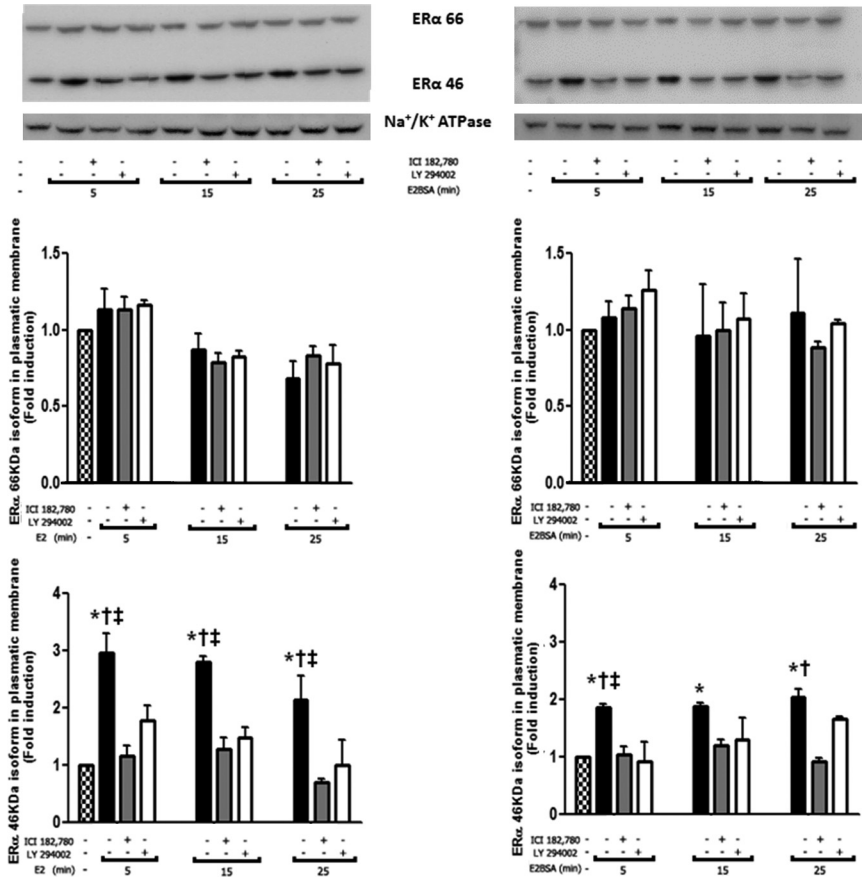

Figure 4. Changes in Plasma Membrane Total Amount of ERα66 and ERα46 (A and B, respectively) Levels of 0.1% Vehicle (−E2/E2BSA), 10 nM E2, or 10 nM E2BSA, Alone or in Combination with 10 nM ICI 182 780 or 10 μM LY 294002, That Were Both Administered 60 Minutes before the Hormonal Treatment The histogram shows the densitometry analysis of the Western blots. Values are means ± SEM of 3 independent experiments, and they are normalized with respect to the control value (vehicle). Significant differences ($P \leq 0.05$) are represented with respect to vehicle (*), with respect to combination with ICI 182 780 (†), or with respect to combination with LY 294002 (‡).

also limited the interaction between ERα and p85α to baseline levels.

Discussion

It is well known that E2 exerts its effects in breast cancer by both genomic and nongenomic mechanisms. Over the last 15 years, novel nongenomic mechanisms of steroid hormones have been gaining in importance, and reports of rapid effects of steroid hormones in both in vitro and in vivo experimental models have been increasing in number. Specifically, numerous studies have appeared concerning the interaction in a variety of models between the insulin-signaling pathway and steroid hormones (22, 41, 42). Collectively these studies have postulated that steroid hormones, acting through specific receptors in traditional insulin-dependent tissues such as skeletal muscle or adipose tissue, can increase the insulin sensitivity of target tissues and, by extension, stimulate cell glucose uptake during both chronic (20) and acute hormone exposure (43).

Because E2 stimulates cell proliferation in ERα-positive breast cancer cells (1) and because tumor cells have high energy requirements due to their high proliferation rate (in addition to metabolic features such as the Warburg effect) (44–46), we asked whether E2 stimulates glucose uptake in these cells via the PI3K/Akt signaling pathway cascade, which would benefit tumor cell proliferation. We used the fluorescent glucose analog 2-NBDG to determine whether E2 caused a rapid increase in the glucose uptake of MCF-7 temporal cells. Both E2 and E2BSA increased the 2-NBDG uptake by rapid, nongenomic actions (Figure 1). The ERα dependence of this effect was demonstrated by cotreatment with ICI 182 780. The fact that similar results were obtained with E2 and with E2BSA implies that this effect is triggered, at least in part, by E2-specific membrane-localized receptors.

Considering the relationship between E2 and PI3K/Akt signaling pathway, we postulated that GLUT4 might have an important role in MCF-7 glucose uptake, even though all published work on this topic has focused on GLUT1 as the GLUT with relevance on breast glucose uptake. Consequently, the following could be the most important result of this study. Surprisingly, both E2 and E2BSA caused a significant increase in the presence of GLUT4 in the plasma membrane of MCF-7 cells without any change in the total intracellular amount of this insulin-dependent GLUT (Figure 2). This suggests that the PI3K/Akt signaling pathway cascade could have an important role in this cell line in the regulation of both glucose metabolism specifically, and cell nutrition in general. These data invite a novel perspective on the metabolism of estrogen-dependent breast cancer cells, and it could afford a basis for the development of novel strategies to impair their nutrition. Based on its complete reversal by ICI 182 780, this effect appears to be mediated by specific ERs that, given that E2BSA and E2 were equally effective in this context, are at least partially

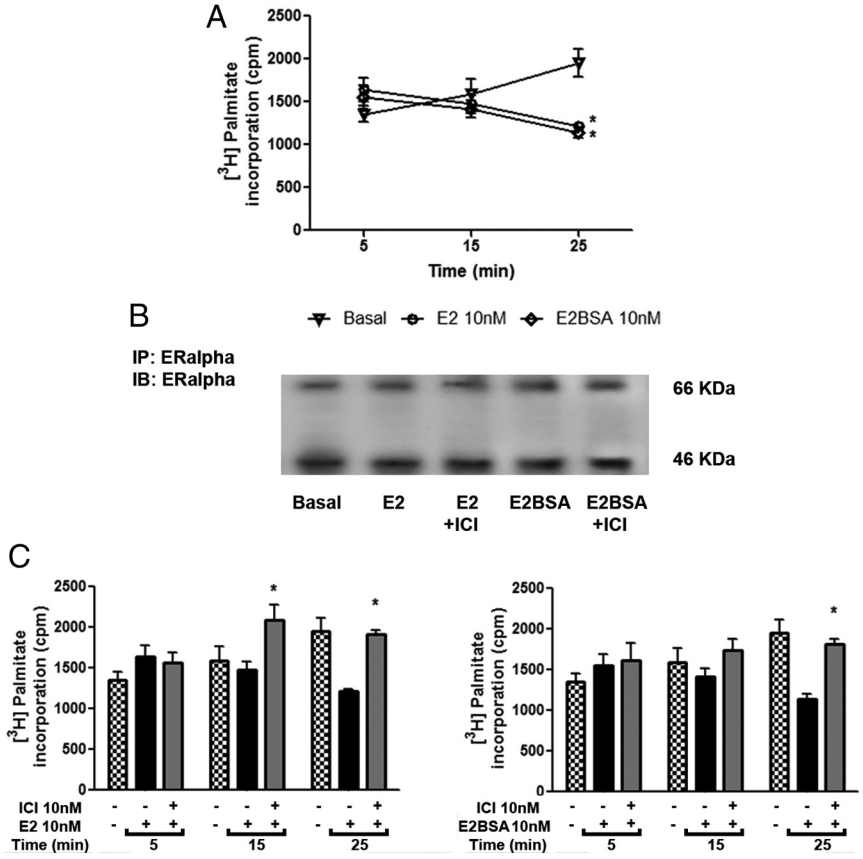

Figure 5. Effect of E2/E2BSA on ERα Palmitoylation A, Time response to hormonal treatment. [^{3}H]palmitate was incorporated to cells during 5 to 25 minutes of hormonal stimulation. Data are the means of 5 different experiments ± SEM. Significant differences ($P \leq .05$) are represented with respect to vehicle (*) and with respect to the same treatment with 5 minutes treatment (‡). B, Western blot analysis for the effectiveness of the immunoprecipitation and hormonal treatment during 25 minutes did not affect the total amount of ERα immunoprecipitated. C, Effect of ICI 182 780 on both E2 and E2BSA stimulation; the ERα antagonist was added at the same time as the [^{3}H]palmitate and 30 minutes before the hormonal treatment. Data are represented as the mean of 5 different experiments ± SEM. Significant differences ($P \leq .05$) are represented with respect to vehicle (*) and with respect to combination with ICI 182 780 (†). IB, immunoblotting; IP, immunoprecipitation.

located in the plasma membrane. Moreover, the dependence of GLUT4 translocation on PI3K signaling is implied by its inhibition by LY 294002. It may be speculated that the pronounced estrogen dependence of this tumor cell line is related to the involvement of the PI3K/Akt signaling pathway cascade in this context and underlies the increased importance of GLUT4 in their metabolism. Bearing in mind the primacy of GLUT1 in the basal nutrition of the mammary gland, we determined the effect of our treatment on the location of this transporter in the plasma membrane and its total cellular levels (Figure 3) and detected no effect. This result excludes a crossed effect between both GLUTs at the same time that provides an unequivocal causal link between the increased GLUT4 in the plasma membrane and the 2-NBDG uptake mediated by E2, therefore confirming the significance of GLUT4 in the estrogen-dependent metabolism of this cell line. In order to totally confirm the role of GLUT4 on the progression of these cells, and accordingly to the suggestion of the reviewers, we think that we must address an experiment in which GLUT4 expression is knocked down with small interfering RNA. Actually we have started this project, and we expect the results to be the reason for a new publication.

In our aim to clarify the role of plasma membrane ERs in this phenomenon, we verified their presence in the plasma membrane. The involvement of the splicing isoforms of ERα (ERα46 and ERα36) in the triggering of nongenomic effects is clearly proved in the literature (11, 47, 48). In fact it is proved that, after E2 stimulation, the splicing isoform ERα46 associates in the caveola with several kinases, activating signaling pathways such as MAPK or Akt in in vitro breast cancer models (49). We could not verify the presence of the ERα36 isoform, because until now most of the studies have used no commercial antibodies (50). Actually, we could verify the presence of both isoforms previously described (51) and demonstrated that ERα46, but not ERα66, was mobilized to the plasma membrane in response to both E2 and E2BSA (Figure 4). We observed a significant increase in the presence of ERα46 in the plasma membrane in response to E2 treatment, which possibly serves to amplify the nongenomic effects on glucose homeostasis described above. Collectively these data suggest a key role for ERα46 in mediating these nongenomic effects through the PI3K/Akt signaling pathway, which will be confirmed in future studies also encompassing the ERα36 isoform. This fact just confirms what has been previously observed in other experimental models (6, 52). Palmitoylation of ERα Cys 447 appears to be essential for its mobilization in the proximity of the plasma membrane and its interactions with other membrane proteins (14). We observed in our MCF-7 cell model, in the absence of hormonal stimulation, that the incorporation of [^{3}H]palmitate into ERα increased over a 25-minute period. In contrast, both E2 and E2BSA decreased the palmitoylation of ERα below basal levels, and addition of ICI 182 780 restored palmitoylation to basal

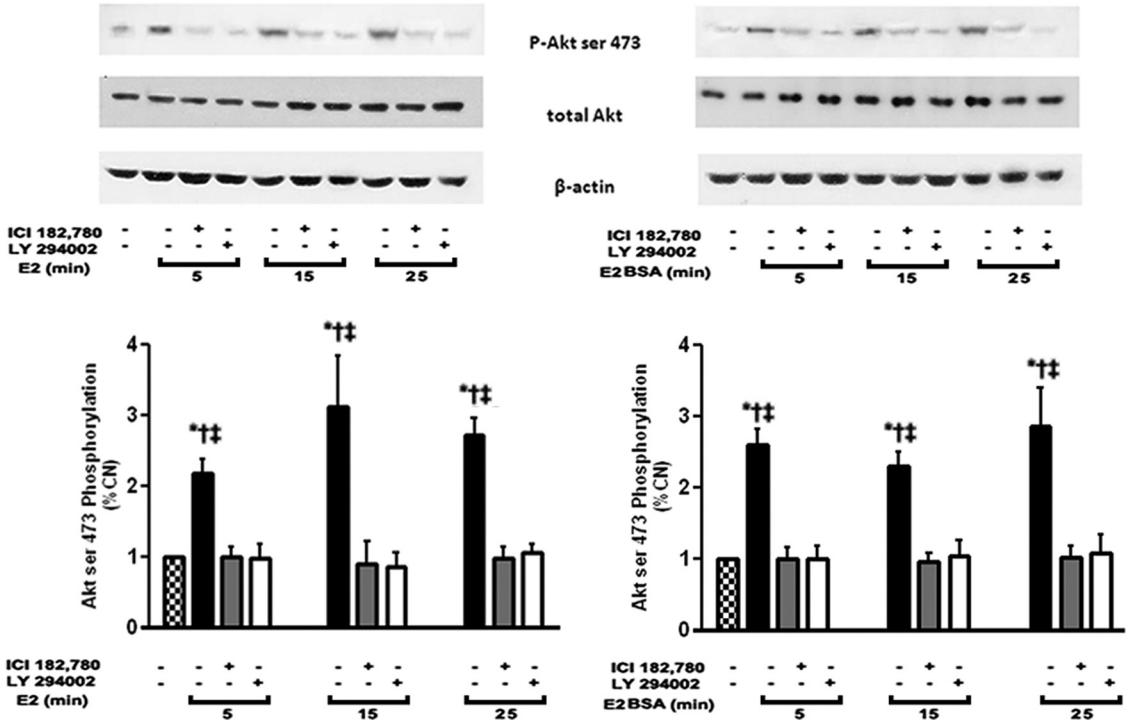

Figure 6. P-Akt Serine 473 Levels of 0.1% Vehicle (−E2/E2BSA), 10 nM E2, or 10 nM E2BSA, Alone or in Combination with 10 μM ICI 182 780 or 10 μM LY 294002, That Were Both Administered 60 Minutes before the Hormonal Treatment The histogram shows the densitometry analysis of the Western blots. Values are means ± SEM of 3 independent experiments, and they are normalized with respect to the control value (vehicle). Significant differences ($P \leq .05$) are represented with respect to vehicle (*), with respect to combination with ICI 182 780 (†), or with respect to combination with LY 294002 (‡).

levels (Figure 5). As shown in previous studies upon E2 stimulation (53), the ERα may undergo depalmitoylation to dissociate from these docking proteins and interact with other proteins in the proximity of the plasma membrane to trigger its nongenomic effects.

E2 and E2BSA appeared to increase recruitment of the ERα46 isoform to the plasma membrane (Figure 4) but, at the same time, seemed to enhance the depalmitoylation of ERα to promote its interaction with the kinases situated near the plasma membrane, ultimately triggering its nongenomic effects (Figure 5). Although these effects appear to be contradictory, it has been shown that ERα lacking the A/B domain, the same domain absent from the ERα46 isoform (49), is recruited to the plasma membrane by palmitoylation in an E2-dependent manner in human cancer cells (53). Thus we hypothesize that in the plasma membrane we can define different populations of ERα with different roles. The plasma membrane levels of one ERα46 population increase in response to stimulation by E2 or E2BSA, reaching a maximum after 5 minutes of treatment, after which they undergo depalmitoylation and release from the plasma membrane to interact with proximal kinases. A second population of ERα is directly anchored to the plasma membrane in basal conditions, and it seems that its presence there is not conditioned by the hormonal treatment. The former population is therefore a "dynamic population" the presence of which in the plasma membrane is regulated by the hormonal treatment and is represented in results of the palmitoylation assay. The results in Figure 4 may represent the contribution of both populations (the dynamic and the anchored), which would explain the relative increase in ERα46 with respect to basal conditions, as demonstrated by Western blotting.

As described elsewhere (35, 54), we also observed that treatment with E2, but also with E2BSA, improved an ERα and PI3K-dependent Akt activation (Figure 6). All of our data suggest a central role for PI3K in all the effects observed, as confirmed with LY 294002 cotreatment. We examined the interaction between p85α, the regulatory subunit of PI3K, and tyrosine-phosphorylated sites of IRS-1 as a key step in GLUT4 mobilization induced by insulin (55). We found a small basal interaction between these two molecules, but no effect of treatment with either E2 or E2BSA (data not shown). For this reason, we developed a new hypothesis in order to explain PI3K activation. Considering the central role of ERα in MCF-7 cells, and also previously described by Simoncini et al (39), we examined the ERα-p85α interaction as a possible step preceding Ser473 phosphorylation of Akt. We confirmed that treatment with E2 or E2BSA in our experimental

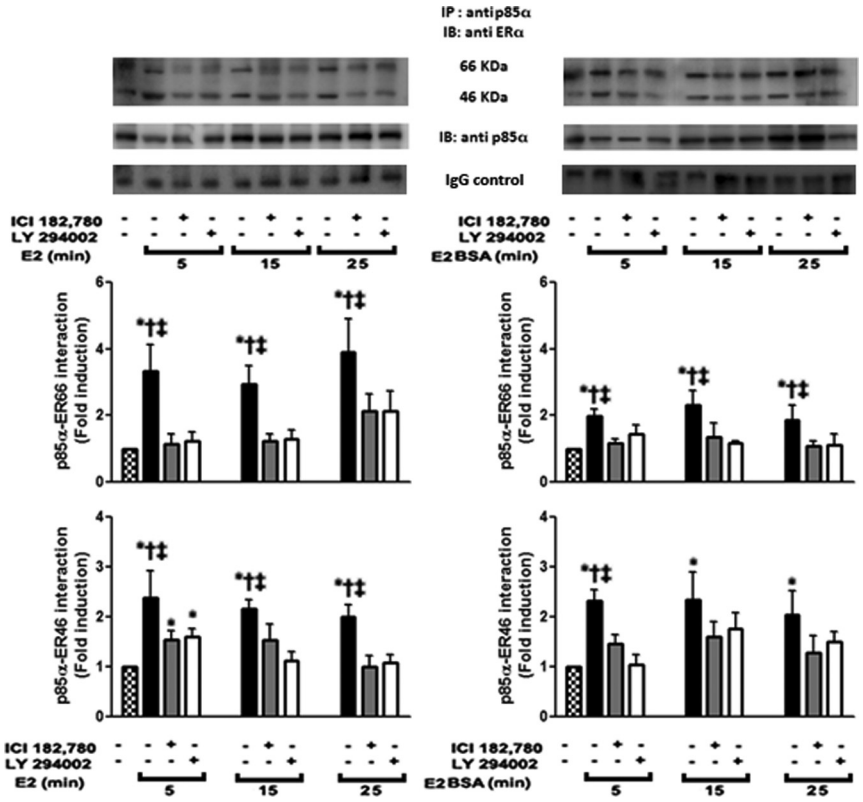

Figure 7. Interaction between p85α and ERα of 0.1% Vehicle (−E2/E2BSA), 10 nM E2, or 10 nM E2BSA Alone or in Combination with 10 nM ICI 182 780 or 10 μM LY 294002, That Were Both Administered 60 Minutes before the Hormonal Treatment The histogram shows the densitometry analysis of the Western blots. Values are means ± SEM of 3 independent experiments, and they are normalized with respect to the control value (vehicle). Significant differences ($P \leq .05$) are represented with respect to vehicle (*), with respect to combination with ICI 182 780 (†), or with respect to combination with LY 294002 (‡). IB, immunoblotting; IP, immunoprecipitation.

model increased the interaction of both analyzed isoforms of the ER with p85α (Figure 7). Because we are unable to distinguish between the isoforms, we cannot know which isoform has the more important role in this activation, and future studies will be needed to clarify this interaction. These observed effects are inhibited by ICI 182 780; therefore we can say they are mediated by specific receptors that are located, at least in part, in the plasma membrane. Nevertheless, it is intriguing that LY 294002, although it is an inhibitor of p110 catalytic activity (31) and has no theoretical effect on p85α, inhibited the interaction between ERα and p85α in both hormonal treatments. Further studies are needed to elucidate the molecular mechanisms underlying these complex regulatory systems.

Based on our results, we can conclude that E2 acts in MCF-7 cells through rapid mechanisms triggered by specific receptors located, at least in part, in the plasma membrane and, probably in subcellular structures such as caveola (49, 56), inducing the ER to interact with key members of the insulin signaling cascade. This effect follows an induction of metabolic responses pursuant to greater energy needs arising from an increased proliferation rate. Specifically, in this case free or BSA-coupled E2, triggering the ERα-p85α interaction, is able to activate Akt by phosphorylation of Ser473, which could stimulate the recruitment of GLUT4 to the plasma membrane and, consequently, appears to enhance glucose uptake in this in vitro model. This would presumably afford these cells a proliferative advantage, taking into account the fact that proliferating cells are characterized by a higher energy demand (44).

On the basis of these results, we conclude that E2 not only enhances proliferation in ERα-positive breast cancer cells, but it also triggers nongenomic effects that provide the tumor cells molecular mechanisms that afford them to solve the energy requirements resulting from their increased proliferation rate. Once better understood, these processes may have clinical relevance, even more if we consider that the mean survival time of patients with tumors exhibiting high glucose utilization is significantly shorter than that of patients with tumors with lower glucose utilization (57, 58), in several tumor types. Of course, more studies are needed to fully understand the complex interaction in tumors between estrogen and the PI3K/Akt signaling pathway cascade, potentially enabling us to develop novel therapeutic targets.

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