Estrogen increases Nrf2 activity through activation of the PI3K pathway in MCF-7 breast cancer cells

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\textbf{Abstract}

The actions of the transcription factor Nuclear factor erythroid 2-related factor (Nrf2) in breast cancer have been shown to include both pro-oncogenic and anti-oncogenic activities which is influenced, at least in part, by the hormonal environment. However, direct regulation of Nrf2 by steroid hormones (estrogen and progesterone) has received only scant attention. Nrf2 is known to be regulated by its cytosolic binding protein, Kelch-like ECH-associated protein 1 (Keap1), and by a Keap1-independent mechanism involving a series of phosphorylation steps mediated by phosphatidylinositol 3-kinase (PI3K) and glycogen synthase kinase 3 beta (GSK3\textbeta). Here, we report that estrogen (E2) increases Nrf2 activity in MCF7 breast cancer cells through activation of the PI3K/GSK3\textbeta pathway. Utilizing antioxidant response element (ARE)-containing luciferase reporter constructs as read-outs for Nrf2 activity, our data indicated that E2 increased ARE activity \textasciitilde 14-fold and enhanced the action of the Nrf2 activators, tertiary butylhydroquinone (tBHQ) and sulforaphane (Sul) 4 to 9 fold compared with cells treated with tBHQ or Sul as single agents. This activity was shown to be an estrogen receptor-mediated phenomenon and was antagonized by progesterone. In addition to its action on the reporter constructs, mRNA and protein levels of heme oxygenase 1, an endogenous target gene of Nrf2, was markedly upregulated by E2 both alone and in combination with tBHQ. Importantly, E2-induced Nrf2 activation was completely suppressed by the PI3K inhibitors LY294002 and Wortmannin while the GSK3\textbeta inhibitor CT99021 upregulated Nrf2 activity. Confirmation that E2 was, at least partly, acting through the PI3K/GSK3\textbeta pathway was indicated by our finding that E2 increased the phosphorylation status of both GSK3\textbeta and Akt, a well-characterized downstream target of PI3K. Together, these results demonstrate a novel mechanism by which E2 can regulate Nrf2 activity in estrogen receptor-positive breast cancer cells and suggest that patients’ hormonal status through this activity may play a significant role in some therapeutic outcomes.

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Introduction

Nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that integrates cellular stress signals and responds by directing various transcriptional events through its binding to antioxidant response elements (ARE) within promoter regions of Nrf2-regulated genes [1–4]. Among more than 100 genes that are regulated by Nrf2 are numerous detoxifying enzymes, including NAD(P)H-quinone oxidoreductase 1 [5], glutathione S-transferases [5], heme-oxygenase 1 (HO-1) [6], γ-glutamylcysteine synthetase [7] and glucuronosyl transferases [8]. The net result of this coordinated gene induction profile is an increased capacity of cells to metabolize and eliminate chemical insults and maintain cellular redox homeostasis. This action has given rise to the notion that Nrf2 has tumor suppressor functions and that its activity can, in some circumstances, antagonizes carcinogenesis. For example, the Nrf2 activator sulphoraphane (Sul) has been shown to inhibit the growth of human breast cancer cells in vitro and suppress the growth and metastasis of orthotopically transplanted breast cancer cells in female athymic mice [9]. In prevention studies using genetic models of carcinogenesis, another class of Nrf2 activators, synthetic cinnamate tripenoid derivatives, were shown to delay breast cancer development in Brca1-mutated mice [10]. In contrast, more recent genetic studies of human breast tumors have indicated that the actions of Nrf2 can also be oncogenic [11,12] and cause resistance to chemotherapy. For example, chronic activation of the Nrf2–ARE pathway has been observed in breast cancer cells with acquired resistance to tamoxifen [13] and doxorubicin [14]. Thus, data suggest that the role(s) played by Nrf2 in cancer development and response to treatment is highly complex. To this end, the circumstances in which Nrf2 either promotes or is protective against oncogenic events need further investigation.

A well-characterized mechanism of Nrf2 regulation is through its cytosolic binding protein, Kelch-like ECH-associated protein 1 (Keap1) [15]. Under basal conditions, Nrf2 is kept transcriptionally inactive through binding with Keap1, which targets Nrf2 for proteasomal degradation. In the presence of drugs or oxidative stress, Keap1 dissociates from Nrf2 which allows it to translocate to the nucleus to function as a transcription factor. In more recent studies, Nrf2 has also been shown to be regulated by Keap1-independent mechanisms involving the PI3K–Akt–GSK3β signaling pathway. To this end, the activity of GSK3β is negatively regulated by the action of Akt-catalysed phosphorylation which can reduce the exportation of Nrf2 from the nucleus [16,17] as well as suppress its ubiquitylation [18,19]. Both of these actions then lead to alteration of nuclear Nrf2 protein and downstream target genes. In the present study, we report that estrogen can increase Nrf2 activity through activation of the PI3K/Akt/GSK3β pathway in human breast cancer cells. Our results suggest that hormonal regulation of Nrf2 activity in breast cancer may be an important consideration during various stages of treatment and long-term patient care.

Materials and methods

Cell cultures and chemicals

The human breast cancer cell lines MCF7 cells were obtained from Dr. Thomas Klonisch (University of Manitoba). MDA-MB-231 and T47D cells were purchased from ATCC. All cultures were grown in complete medium: Dulbecco’s modified Eagle’s medium (DMEM, Cellgro, Manassas, VA) containing 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 1 mM L-glutamine and 1 mM HEPES. Estradiol (E2), progesterone (P4) and medroxyprogesterone acetate (MPA) (Sigma Chemical Co., St. Louis, MO) were diluted in absolute ethanol to a stock concentration of 100 μmol/L and then diluted to the indicated concentration in complete medium for the experiments. tert-butylinhdroquinone (tBHQ, Sigma), Sulforaphane (Sul, Sigma), ICI 182,780 (Sigma), LY294002 (Sigma) and CHIR-99021 (CT99021, Selleck Chemicals, Houston TX) were utilized at the concentrations indicated. Vehicle controls contained the same final solvent concentration. ER antagonists methyl-piperidino-pyrazole dihydrochloride (MPP) and 2-phe- ny1-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a] pyrimidine (PHTPP) were purchased from Tocris Bioscience, United Kingdom.

Luciferase reporter assay

The antioxidant response element (ARE) reporter plasmid was generated by cloning four ARE motifs [20] upstream of the TATA sequence in pGL2 as described previously [21]. It was used in our experiment as read-outs for Nrf2 activity. β-galactosidase (β-gal) reporter plasmids were used as transfection controls. The ERE- and PRE-Luc constructs have been described [22,23]. The AP-1-Luc reporter plasmid was purchased from Agilent Technologies (Santa Clara, CA). Cells were cultured in E2-depleted medium (phenol red-free RPMI-1640 containing 10% charcoal-stripped, steroid-depleted FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin) in 12-well plate for 2–3 days before performing the assays. 1 nM E2 or vehicle control was then added into wells one day before transfection (“priming period”). Transfection was performed using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). 6–8 h after transfection, treatments as indicated were added into wells in duplicates for each treatment condition. After 16–24 h additional incubation, cells were rinsed with PBS twice and lysed to measure the luciferase level using the Luciferase assay kit (Promega, Madison, WI). Data were normalized to the co-transfected β-gal activity and expressed as values (± SEM) relative to that obtained in vehicle-treated controls.

Real-time PCR analysis

For mRNA quantitation, reverse transcription was used to synthesize cDNA from RNA template as described previously [24]. For real-time quantitative PCR, we used 2 × SYBR Green Supermix (Bio-Rad, Hercules, CA) and followed the vendor’s guidelines with some modifications. A total reaction volume of 20 μl contained 10 μl 2 × SYBR Green, 1 μl 50 mM MgCl2, 1 μl primer mix and 8 μl diluted cDNA. Samples were processed using Opticon Monitor3 software (Bio-Rad–Hercules, CA) under the following conditions: one denaturation cycle of 95 °C, 5 min followed by 40 amplification cycles of 95 °C, 30 s, 60 °C, 30 s, and 72 °C, 30 s. PCR was run in an Opticon2 real-time thermocycler (Bio-Rad). The data were analyzed after normalization to actin as previously described [25]. Primer sequences utilized were: HO-1, sense (5’-CCAGGGCC- CAGCA-3’), antisense (5’-AAAGGCTCTGCGCC-3’); actin, sense (5’-ACATGCTACTGGGCTT-3’), actin, sense (5’-TATGAGCTCCGACCTTTTTCTGTC-3’). Melting
curve analysis of each sample revealed sharp, single amplicon peaks and these were supplemented with agarose gel electrophoresis of randomly selected samples to confirm the success of amplification reactions and the size of the amplicon.

**Western-blot analysis**

Western-blot analysis was performed on whole-cell extracts that were obtained by direct dissolution of cell pellets in Cell Extraction Buffer (Invitrogen), followed by protein determination using a bicinchoninic acid protein assay kit (Sigma Chemical Co.). Protein (20 μg) from control cells or treated cells was loaded on the 10% SDS-PAGE gel, then transferred to nitrocellulose membrane and blocked with 4% bovine albumin in PBS. Endogenous HO1 was detected using the rabbit polyclonal anti-HO1 antibody (1:1000, Assay designs - Enzo Life Sciences, Farmingdale, NY), then incubated with the secondary antibody linked to horseradish peroxidase. Phosphorylated form of Akt and total Akt was detected using the rabbit anti-pAkt antibody (1:1000), and the mouse monoclonal anti-Akt (pan) (1:1000), respectively (Cell Signaling, Danvers, MA). Phosphorylated form of GSK3β and total GSK3β was detected using the rabbit anti-p-GSK3β (Ser9) antibody (1:1000) and the mouse monoclonal anti-total GSK3β (1:1000), respectively (Cell Signaling). The immunoreactive bands were visualized by the Enhanced Chemiluminescence System (Amersham Biosciences AB, Uppsala, Sweden). Blots were washed, stripped, reprobed with an anti-β-actin antibody, and developed in an identical manner for assessing β-actin protein levels to ensure even loading.

**Statistical analyses**

All experiments shown were performed for a minimum of three times. SPSS software was used for data analysis, and the data were expressed as mean ± SEM. Significant differences between treatment groups were accepted when Student’s t test (two-tailed analyses) yielded P < 0.05 between the groups. The data shown in some figures (e.g. photographs of Western blots) are from a representative experiment, which was qualitatively replicated in at least three independent experiments.

**Results**

**Effects of estrogen and progesterone on Nrf2 activity in MCF7 cells**

Following culture of the cells in estrogen-free medium for 3 days, the functionality of endogenous estrogen receptors (ERs) in our MCF7 cell line was demonstrated in transient transfection assays utilizing an ERE-Luc reporter plasmid (Fig. 1A). Induction of

![Fig. 1](image-url)

Fig. 1 – E2 increases Nrf2 activity in MCF7 cells. MCF7 cells were cultured in E2-depleted medium for 2–3 days and then primed for 1 day with 1 nM E2 or vehicle control before being transfected with (A) ERE-Luc, (B, D) PRE-Luc, (C, D) ARE-Luc, or (D) AP-1-Luc reporter plasmids. After 6–8 h of transfection, the cells were treated for 24 h as indicated on the abscissas of each figure with vehicle control (C), E2 (10 nM), P4 (100 nM), tBHQ (12.5 μM) or sulforaphane (Sul, 1 μM). Luciferase activity was normalized to β-gal activity as described in “Materials and Methods”. Values represent mean ± SEM of at least three independent experiments for each condition and are expressed relative to control cultures. In all figures, ∗ represents significant increase from controls. (C) Values that are labeled with identical letters also showed significant differences.
functional progesterone receptor (PRs) in the cells was demonstrated by transfection of a PRE-Luc reporter construct and P4 treatment following incubation (“priming”) of the cells with 1 nM E2 for 24 h. Only a slight increase of PR activity was seen in cells that were not primed with E2 (Fig. 1B). Thus, this 24 h priming protocol was used to assess hormonal regulation of Nrf2 activity. Utilizing an ARE-Luc reporter plasmid, treatment with E2 resulted in >14-fold induction of luciferase activity compared with estrogen-depleted cultures (Fig. 1C). In parallel transfection assays, E2 had no effect on luciferase activity generated either by the PRE-Luc construct or by an AP-1-Luc reporter, demonstrating specificity to the action of E2 on ARE (Fig. 1D). Treatment with tBHQ and sulforaphane (Sul), known inducers of Nrf2 activity, increased ARE-Luc activity approximately 8- and 6-fold, respectively, compared to estrogen-depleted conditions. The addition of E2 further enhanced the actions of tBHQ and Sul approximately 9- and 4-fold, respectively, compared to cultures treated with the Nrf2 activators as single agents (Fig. 1C).

The effects of E2 on Nrf2 activity were shown to be an ER-mediated phenomenon since E2 upregulation of ARE-Luc was markedly inhibited by the ER antagonist ICI 182,780 (Fig. 2A). As expected, ARE-Luc activity induced by tBHQ alone was not affected by ICI 182,780. These results were supported by parallel experiments utilizing the ER-negative breast cancer cell line MDA-MB-231 that showed no effects of E2, either alone or in combination with tBHQ, on ARE-Luc activity (Fig. 2B). In contrast, in another ER positive cell line, T47D, estrogen and tBHQ had similar effects on Nrf2 activation as we observed in MCF7 cells. To clarify which estrogen receptor mediates the effect of E2 on Nrf2 activation, we assessed Nrf2 activity in the presence of selective ERα and ERβ antagonists: MMP [26] and PHTPP [27]. As shown in Fig. 2C, the ERα antagonist MMP significantly blocked E2-induced Nrf2 activity. The synergistic effect of E2 and tBHQ on Nrf2 activation was also suppressed by MMP. In contrast, the ERβ antagonist PHTPP had no inhibitory effect on Nrf2 induced by E2 treatments, either alone or in combination with tBHQ. This data demonstrate that ERα, but not ERβ is essential for E2-mediated stimulation of Nrf2 in MCF7 cells.

To confirm the physiological relevance of the reporter gene assays, we assessed the effects of our treatment protocol on mRNA and protein levels of HO1, an endogenous target gene of Nrf2 [6]. As shown in Fig. 3A, E2 alone upregulated mRNA and protein levels of HO1. As in the reporter assays, we noted dramatic further increases in HO1 mRNA and protein levels in E2+tBHQ-
treated cells compared with tBHQ or E2 as single-agents. Qualitatively similar results were observed when tBHQ was replaced with Sul (Fig. 3B).

To determine if progesterone can oppose the action of E2 in inducing Nrf2 activity, P4 (100 nM) and MPA (100 nM) were added to ARE-Luc-transfected cultures following the 24 h E2-priming period. Fig. 4 shows that P4 and MPA inhibited E2-induced ARE activity by 18% and 30%, respectively. Quantitatively similar inhibitory effects were seen with both progestogens on Nrf2 activity resulting from combination treatment with E2+tBHQ (Fig. 4). By themselves, neither progestogen had any effect on reporter gene activity or on Nrf2 induction by tBHQ as a single agent (data not shown).

The PI3K/Akt/GSK3β pathway is involved in E2 stimulation of Nrf2

The serine/threonine kinase GSK3β has been shown to mediate ubiquitylation of Nrf2, resulting in decreased Nrf2 activity [18,19]. Phosphorylation of GSK3β via PI3K/Akt signaling inhibits the function of this kinase which then promotes an increase in the actions of Nrf2 [18]. As such, we hypothesized that E2 activation of PI3K/Akt signaling and subsequent phosphorylation of GSK3β may be involved in its ability to increase Nrf2 activity and induce HO1 in MCF7 cells. To test this hypothesis, we examined the effects of the PI3K inhibitors Ly294002 and Wortmannin [28] on reporter gene activity and HO1 protein expression. As shown in Fig. 5A, Ly294002 (10 μM) and Wortmannin (100 nM) completely eliminated E2-induced ARE-Luc activity. In contrast, these PI3K inhibitors were significantly less effective in suppressing ARE-Luc activity induced by tBHQ (~50% inhibition). Fig. 5B indicates that Ly294002 also blocked induction of HO1 by E2. Consistent with our hypothesis that E2-mediated inhibition of GSK3β function can promote Nrf2 activity in our cells, experiments utilizing the GSK3β inhibitor CT99021 [29] showed that its effects were, like E2, stimulatory for Nrf2, both alone and in combination with tBHQ (Fig. 6).

E2 stimulates phosphorylation of Akt and GSK3β

Having shown that the PI3K pathway is essential for E2 induction of Nrf2 and HO1 expression, we directly assessed the ability of E2 to upregulate PI3K signaling. Since Akt is an established target substrate for PI3K, the degree of phosphorylation of Akt has been used as an excellent correlate of PI3K activity in many cell systems [30,31]. Fig. 7A indicates that expression of phospho (p)-Akt rapidly increased in E2-treated cells while levels of total Akt protein were unaffected. Phosphorylation of GSK3β at ser9 is exclusively mediated by the action of Akt [32,33]. Therefore, we utilized anti-p-GSK3β (ser9) specific antibody in Western blot analysis of cells undergoing our treatment protocol to further assess the ability of E2 to influence Nrf2 through the Akt/GSK3β pathway. Similar to results obtained for p-Akt, E2 treatment showed a rapid increase of the p-GSK3β (ser9) species without upregulation of total GSK3β levels (Fig. 7B). No significant effects
Discussion

Nrf2 has become the subject of widespread interest due to its impact on the development and growth of many cancers including breast cancer and other estrogen-driven tumors [34–36]. Although it is well established that estrogen exposure is a major risk factor in such malignancies, the relationship between estrogen and Nrf2 has received only scant attention and, in particular, mechanistic information regarding hormonal regulation of Nrf2 has been lacking. Moreover, there is conflicting data in the literature regarding the ability of estrogen to regulate Nrf2 activity; depending on the species and cell types investigated, both inhibition and stimulation of Nrf2 by E2 have been reported [37,38]. A mechanistic understanding of these differences in the effects of E2 is unknown. Possible explanations may be related to the balance between cell-specific factors and co-factors that drive suppression versus stimulation of Nrf2. In this regard, E2-mediated inhibition of Nrf2 activity has been shown to involve a physical association between ERα and Nrf2 that impedes Nrf2–ARE interactions [37]. In contrast, E2 stimulation of Nrf2 appears to be dependent on PI3K and its downstream effectors (e.g. Akt, GSK3β) that can increase the protein level of Nrf2 in the nucleus (38 and present study). When the combination of these two mechanisms come into play, the overall effects of E2 on Nrf2 activity are likely to be variable and dependent on the cell-specific concentrations of cellular proteins/co-factors involved in those respective actions. Our results showed that in MCF7 cells, estrogen can upregulate Nrf2 activity through activation of the PI3K/Akt pathway in an ER-dependent manner. We found that estrogen had the same effect on Nrf2 activation in T47D cells as we observed in MCF7 cells. Recent reports using primary murine mammary epithelial cells [39] and an estrogen-responsive neuronal cell line [38] also demonstrated that estrogen induces Nrf2 activity which is dependent on PI3K–Akt activation. Together, those data illustrate the generalized nature of our findings regarding the pathway by which E2 can modulate Nrf2 activity in E2-responsive cell types.

The classical actions of receptor-bound E2 to function as a nuclear transcription factor have been thoroughly studied. However, the actions of E2 outside the nucleus remain controversial. In the cardiovascular system, ER was shown to mediate the

Fig. 5 – PI3K is required for E2 stimulation of Nrf2. (A) ARE-Luc reporter assays were performed in MCF7 cells that were treated for 24 h as indicated with vehicle control, E2 (10 nM), tBHQ (12.5 μM), or E2+tBHQ in the absence or presence of the PI3K inhibitors Ly294002 (10 μM) or Wortmannin (100 nM). Values represent mean±SEM of at least three independent experiments for each condition and are expressed as a percentage of values obtained in the absence of the inhibitors. Average vehicle control values relative to the corresponding treatment condition is indicated by the horizontal dotted lines on the first bar in each treatment group. (B) Analysis by Western blotting of HO1 protein expression in MCF7 cells that were treated as in (A) in the absence or presence of Ly294002 (10 μM). A representative example of three independent experiments is shown in the inset. Quantitation was obtained by densitometry and results expressed as mean±SEM of normalized H01 band values (i.e. H01/actin) of treated cultures relative to vehicle controls (C) without inhibitor. *, significance difference from similarly-treated cultures not containing inhibitor.

on p-Akt or p-GSK3β were noted by treatment with tBHQ either alone or in combination with E2 (data not shown).

Fig. 6 – GSK3β plays a role in regulating Nrf2 activity in MCF7 cells. ARE-luc reporter assays were performed under E2-free (i.e. vehicle control) conditions using the treatment protocol described in legend to Fig. 1. During the final 24 h treatment phase of the assay, the cultures were treated with vehicle control, tBHQ, E2 or tBHQ+E2 in the absence or presence of the GSK3β inhibitor CT99021 as indicated. Values represent the mean±SEM of three independent experiments and are expressed relative to vehicle controls (C). *, significance difference from similarly-treated cultures not containing CT99021.
cellular protective effects of estrogen via activation of the PI3K/Akt pathway in an extranuclear environment [40–42]. The molecular mechanism underlying E2-mediated activation of PI3K in that system was not determined although other studies in endothelial cells suggest that it involves E2–ERα binding to the p85α regulatory subunit of PI3K, leading to its increased activity and subsequent phosphorylation of Thr308/Ser473 of Akt. It is unknown whether E2 works through a similar mechanism to activate PI3K in human breast cancer cells.

Progesterone, by itself, has been shown to regulate a variety of cellular functions both through its cognate PR, and in a PR-independent fashion. Although we confirmed the induction of functional PR in our treatment protocol, neither P4 nor MPA, as single agents, had any effect on Nrf2 activity. On the other hand, in co-treatment protocols, these progestogens were found to modestly inhibit E2 induction of Nrf2 as reflected by ARE-Luc reporter gene activity. This antiestrogenic effect is similar in degree to the antagonistic action of P4 and MPA on some other E2-driven gene activity in these cells [43–45]. The opposing effects of estrogen and progesterone in breast cancer have been attributed, at least partially, to progesterin-mediated downregulation of ER. Previous studies have demonstrated expression of ERα to be inhibited by approximately 50% in progesterone-treated MCF7 cells [46–49].

Inducers of Nrf2 have been shown to work through multiple pathways. In the case of tBHQ, induction of Nrf2 is known to involve Keap1 protein by promoting dissociation of Nrf2–Keap1 [50,51] and increasing Nrf2 protein stability [52]. Reports from isolated studies in other cell types proposed a similar link between E2 effects on Keap1 and modulation of Nrf2 activity [53]. Our results suggest that in MCF7 cells, E2-mediated induction of Nrf2 includes other, Keap1-independent, mechanisms of action. To this end, differences in the ability of 294002 and Wortmannin to inhibit tBHQ- versus E2-induced ARE transactivation (Fig. 5A), combined with the fact that tBHQ had no significant effects on p-AKT and p-GSK3β, suggest non-coincident pathways of action resulting in enhanced Nrf2 activity by these agents. This conclusion is consistent with our finding that E2 induction of Nrf2 is, at least partially mediated through the PI3K/Akt/GSK3β-mediated pathway which has not been implicated in the action of tBHQ.

Importantly, we showed that estrogen not only increased Nrf2 activity alone, but also enhanced the action of tBHQ and Sul 4- to 9-fold compared with cells treated with these compounds as single agents. This synergistic activity is consistent with the contention that the classic Nrf2 activators (tBHQ and Sul) and E2 stimulate Nrf2 through independent mechanisms of action, with the former working through Keap1 and the latter via the PI3K/Akt/GSK3β-mediated pathway. The importance of these combination treatment results is accentuated by the fact that natural Nrf2 activators are contained in many foods and dietary supplements, such as vegetables, fruit, and green tea extracts, as well as in certain drugs and environmental compounds (e.g. turmeric, cuscuta reflexa and arsenic) [54,55]. Thus, Nrf2 activity in estrogen-dependent tissue may be unduly influenced by menstrual-cycle swings in estrogen/progesterone levels depending on food intake or environmental influences. Although Nrf2, in its role as a master regulator of detoxifying enzymes, is generally thought to have protective effects against environmental stressors, increased Nrf2 activity may promote tumorigenesis through stress protection. For example, loss-of-function mutations in human Keap1 have been found in carcinomas of the lung [56,57], gallbladder [58], ovary [59], breast [60], liver and stomach [61]. These mutations result in constitutive Nrf2 activity which may promote tumorigenesis in part through the generation of a more favorable intracellular environment for the survival of tumor cells. In addition, it has been hypothesized that elevated Nrf2 activity triggered by dietary supplements or by chemotherapeutic agents can lead to chemo-resistance of some cancers, such as breast cancer [13,14,62,63]. Thus, for breast cancer

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**Fig. 7** – E2 stimulates phosphorylation of Akt and GSK3β. Total cellular protein was isolated from MCF7 cells and assayed by Western blotting. (A) Time-course of a representative experiment showing the phosphorylated levels of Akt in cells that were treated with 10 nM E2 for the indicated time periods. Total Akt levels did not change and served as loading controls. (B) Time-course of a representative experiment showing the phosphorylated levels of GSK3β under identical conditions as in (A). Total GSK3β levels served as loading controls. (C) Results of a 16 h treatment with 10 nM E2 on the phosphorylated species of Akt (P-Akt) and GSK3β (P-GSK3β). Quantitation was obtained by densitometry and results expressed as E2-induced fold-increase relative to vehicle controls. Columns represent the mean ± SEM of at least three determinations for each treatment condition. *, significant difference from vehicle-treated controls.
patients, the present data may have important implications in terms of monitoring their estrogen/progesterone levels and for adjusting their food and supplement intake during chemotherapy treatment in order to help design better therapies and prevent chemo-resistance. As such, hormonal regulation of Nrf2 activity in breast cancer as described in this report may be an important future consideration during various stages of treatment and long-term patient care.

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