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Estrogen-Induced Mitochondrial Reactive Oxygen Species as Signal-Transducing Messengers[†]

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ABSTRACT: We report here evidence in support of the role of 17 β -estradiol- (E2-) induced mitochondrial (mt) reactive oxygen species (ROS) as signal-transducing messengers. On the basis of monitoring the oxidation of 2',7'-dichlorofluorescein by spectrofluorometry, flow cytometry, and confocal microscopy, we have identified that exposure of E2 triggers the immediate rapid production of intracellular ROS ranging from a 1- to severalfold increase in a variety of cells. E2-stimulated ROS production does not correlate with the activity of the estrogen receptor (ER) in the cells. The ROS is most likely hydrogen peroxide based on its inhibition by antioxidants and catalase and lack of any effects of E2 on O₂^{•−} or NO[•] formation. Confocal microscopy showed that ROS is localized in the perinuclear mitochondria. E2 through anchorage- and integrin-dependent signaling to mitochondria increased ROS generation. Increased intracellular ROS formation identified here for the first time may explain the mechanism of previously reported oxidative damage and subsequent genetic alterations including mutations produced by elevated concentrations of estrogens. The functional consequences of E2-induced ROS formation included the enhanced cell motility as shown by the increase in cdc42 and activation of Pyk2 and the increased phosphorylation of signaling proteins c-jun and CREB. E2-induced ROS activated the binding of three oxidant-sensitive transcription factors: AP-1, CREB, and nuclear respiratory factor 1. In addition to ERs as signaling molecules, our findings further revealed that E2-induced mt ROS also act as signal transducing messengers and suggest new targets for the development of antioxidant-based drugs or antioxidant gene therapy for the prevention and treatment of estrogen-dependent cancer.

The involvement of 17 β -estradiol (E2)¹ in the etiology of human breast cancer is supported by a large body of epidemiologic and experimental evidence (1–7). Recent studies have shown evidence for both an estrogen receptor-

(ER-) dependent and ER-independent rapid, nongenomic E2-induced signal transduction pathway (8). The molecular mechanisms involved in regulating the ER-independent, nongenomic E2-induced signal transduction pathway are not clear although recent evidence suggests that cell surface molecules are a direct target for estrogen action. The rapid stimulation of intracellular reactive oxygen species (ROS) by platelet-derived growth factor, epidermal growth factor, and nerve growth factor suggests that this underlying mechanism for cell growth may be shared with other growth supporting agents, including E2 (9). Therefore, the purpose of this study was to examine whether E2 treatment rapidly induced the production of ROS.

ROS can be generated intracellularly by a variety of enzymes (i.e., NADPH oxidase, xanthine oxidase, lipoxigenase) and through the mitochondrial respiratory chain (10). In this study, we identified mitochondria as a major source of estrogen-induced ROS in breast cancer cells. Mitochondrial (mt) ROS have been shown to activate the MEK/ERK pathway in the growth of endothelial cells (11). Whether mt ROS are involved in signaling pathways that control E2-induced cell proliferation is not known. Therefore, the functional consequences of E2-induced ROS production on the activation of redox-sensitive transcription factors, such

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¹ Abbreviations: AP-1, activator protein 1; CaMKII, calmodulin kinase II; CE, catechol estrogen; CREB, cAMP response element binding protein; DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DPI, diphenyleneiodonium chloride; E2, 17 β -estradiol; ER, estrogen receptor; HBSS, Hank's balanced salt solution; mt, mitochondrial; NAC, N-acetyl-L-cysteine; NO[•], nitric oxide; NOS, nitric oxide synthase; NRF-1, nuclear respiratory factor 1; PAGE, polyacrylamide gel electrophoresis; Pyk2, proline-rich tyrosine kinase 2; ROS, reactive oxygen species; O₂^{•−}, superoxide; VDAC, voltage-dependent anion channel; $\Delta\Psi_m$, mitochondrial membrane potential.

as AP-1, CREB, and nuclear respiratory factor 1, were also explored.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures. Human breast cancer cells MDA-MB-468, MCF7, T47D, and ZR75.1 and the human neuroblastoma cell line SH-SY5Y were obtained from the ATCC and propagated in DMEM/F12 medium without phenol red supplemented with 10% FBS and $1\times$ antibiotic-antimycotic. For experimental purposes, cells were seeded in 10% FBS DMEM/F12 medium and allowed to adhere for 24 h. After cells reached 50% confluency, culture medium was replaced with serum-free DMEM/F12 and allowed to grow for 48 h. Serum deprivation was used to synchronize cells in the G₀/G₁ phase of the cell cycle followed by treatments as described in the figure legends.

Measurement of Reactive Oxygen Species (ROS). Cells were seeded at a concentration of 10×10^3 cells per well in black 96-well plates. Cells were pretreated with various antioxidants and/or mitochondrial inhibitors in Hank's balanced salt solution (HBSS) followed by incubation with $10 \mu\text{M}$ 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR) for 15 min. Cells were then rinsed with HBSS followed with various treatments described in the figure legends. DCFH-DA is a nonfluorescent cell-permeable compound, which is acted upon by endogenous esterases that remove the acetate groups generating DCFH. In the presence of intracellular ROS, DCFH is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The oxidative products were measured with a Tecan Genios microplate reader using 485 and 535 nm as excitation and emission filters, respectively. In addition, DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) and dihydroethidium (Molecular Probes) were used to specifically measure nitric oxide and superoxide anion, respectively. DCFH-DA and DAF-FM diacetate stock solutions were diluted at a 1:1 ratio with Pluronic F-127 (20% w/v). Flow cytometry analysis of cells stained with DCFH-DA was performed to confirm results. MCF7 cells were plated in 6-well plates and pretreated for 1 h with $1 \mu\text{M}$ rotenone followed by 30 min E2 treatment in the presence of DCFH-DA ($10 \mu\text{M}$). DCF fluorescence was measured with a Becton Dickinson FACStar flow cytometer (500 nm excitation, 530 nm emission).

Determination of ROS by Confocal Microscopy. The fluorophores used were DCFH-DA and Mitotracker Deep Red (Molecular Probes). The MCF7 cells were grown on 25 mm diameter coverslips placed in a 6-well plate. The coverslip was removed and placed in a Narishige microincubation chamber holder. The cells were washed twice with HBSS and incubated in 1 mL of HBSS. To the cells were added $2 \mu\text{L}$ of DCFH-DA (1 mM) and $2 \mu\text{L}$ of Pluronic F-127 (Molecular Probes), and the cells were incubated for 15 min at room temperature. To the cells was added $100 \mu\text{L}$ of stock Mitotracker Deep Red ($1 \mu\text{M}$), and the cells were allowed to incubate for 15 min. Cells were washed three times with HBSS and incubated for 15 min more in HBSS followed by treatments. The confocal images were acquired on a Bio-Rad MRC 1024 laser scanning confocal microscope (obtained from Bio-Rad, Hertfordshire, England). The built-in LaserSharp 2000 software from Bio-Rad, Confocal

Assistant software 4.02 (copyrighted by Todd Clarke Brelje), and Metamorph 6 software (Universal Imaging Corp., West Chester, PA) were used for confocal image acquisition and analyses.

Rac-1 and cdc42 Activation Assay. To measure Rac-1 and cdc42 activation, MCF7 cells were lysed in Mg²⁺ lysis buffer provided by the assay kit (Upstate Biotechnology, Inc.), and equal volumes of total lysates were affinity precipitated with $8 \mu\text{L}$ of PAK-1 PBD bound to glutathione agarose beads following the manufacturer's instructions. Precipitates were washed three times with Mg²⁺ lysis buffer and suspended in $25 \mu\text{L}$ of Laemmli's sample buffer. Proteins were separated by 12% SDS-PAGE, transferred onto nitrocellulose membrane, and blotted with anti-Rac-1, clone 23A8, mAb, or anti-cdc42 mAb, followed by a secondary Ab conjugated to horseradish peroxidase (HRP).

Focal Adhesion Kinase Assay. MCF7 (1×10^6) cells were lysed with $500 \mu\text{L}$ of modified RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 1 mM NaVO₄, 2 mM EGTA). Total and phosphotyrosine-activated forms of focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) were separated by SDS-PAGE and subjected to western blot using mAb anti-Pyk2 (BD Transduction Laboratories) and polyclonal antibodies anti-Pyk2 pY⁴⁰², anti-FAK, anti-FAK pY³⁹⁷, and pY⁸⁶¹ (Biosource Int.).

Phosphorylation of CREB and c-jun. Whole cell lysate from MCF7 cells was prepared as described previously (12). Phosphorylated forms of transcription factors c-jun and CREB were separated by SDS-PAGE and subjected to western blot using mAbs anti-phospho-CREB (Ser-133) (Upstate Biotechnology, Inc.) and anti-p-c-jun (Santa Cruz). Phosphorylation was detected by chemiluminescent reaction exposed to X-ray film. Signals were quantified by scanning the film with an HP ScanJet, and the intensity values were obtained using the image analysis program Scanalyze (Michael Eisen, Stanford University). Phosphorylation values for control and treatments were divided by the total level of β -actin protein to ensure equal loading for all lanes. These ratios were then multiplied by 100 to attain the percent increase and decrease in phosphorylation.

Measurement of AP-1, CREB, and NRF-1 Activation. Activation of transcription factors c-jun and CREB in nuclear extracts was measured with the TransAM AP-1 and CREB assay kits (Active Motif). The binding of AP-1 and pCREB to their consensus sequence was detected using a primary anti-phospho-c-jun (Ser-73) and anti-phospho-CREB (Ser-133), followed by a secondary Ab conjugated to HRP. Finally, plates were read with a Tecan Genios plate reader at 450 nm with a reference wavelength of 700 nm.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed with DIG-11-ddUTP 3'-end-labeled probes. The DNA oligonucleotide sequences used for EMSA were as follows: NRF-1 consensus sequence from human mitochondrial transcription factor A (mt TFA) promoter region (NRF-1A, 5'-CGCTCTCCCGCGCCTGCGCCAATT-3'; NRF-1B, 5'-GGGCGGAATTGGCGCAGGCGCGGG-3') (13). Probe labeling and binding reactions were performed following the protocols provided by the DIG gel shift kit manufacturer (Roche). Samples were electrophoresed on a 6% native polyacrylamide gel and transferred to a nylon membrane by electroblotting followed by chemiluminescent detection.

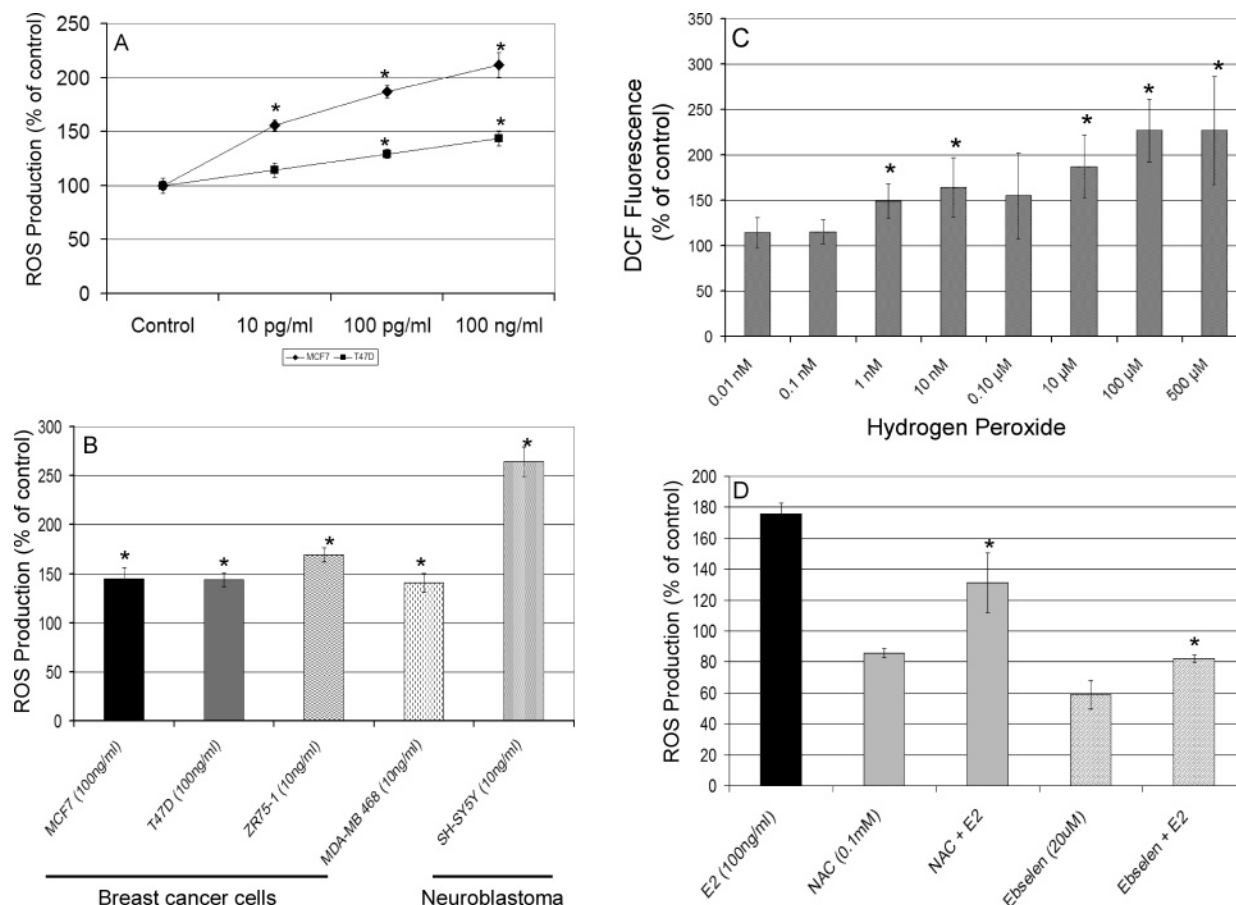


FIGURE 1: Estrogen stimulates the production of ROS in breast cancer epithelial cells. Cells were treated with 0.05% DMSO (control) and E2 at the concentrations shown for 15 min. (A) DCF assay on MCF7 and T47D cells. (B) DCF assay on breast cancer cells and neuroblastoma. (C) DCF assay on MCF7 cells treated with hydrogen peroxide for 15 min. Data from three independent experiments are presented as DCF fluorescence with controls set at 100% (\pm SD). Values that are significantly different from controls ($P < 0.05$) are indicated with an asterisk (*). (D) Antioxidants reduce E2-induced ROS in breast cancer cells. DCF assay on MCF7 cells that were pretreated with NAC or ebselen for 2 h. Data from three independent experiments are presented as ROS production with controls set at 100% (\pm SD). Values that are significantly different from E2 treatment alone ($P < 0.05$) are indicated with an asterisk (*).

Statistical Analysis. Results are expressed as mean \pm SD. Differences between means were evaluated by a two-tailed Student *t* test. ANOVA was used to determine differences between groups.

RESULTS

E2-Induced ROS Production. To evaluate whether E2 can trigger the rapid production of intracellular ROS, a variety of cell lines were seeded in 96-well plates and preincubated with the redox-sensitive fluorescent dye DCFH-DA. The breast cancer cell lines MCF7 and T47D showed a dose-dependent and rapid production of ROS when stimulated with E2 for 15 min (Figure 1A). The estrogen receptor (ER) negative cell line MDA-MB-468 generated an equal amount of ROS as compared to the ER-positive cell lines MCF7, T47D, and ZR75.1 (Figure 1B). We also observed a dramatic increase (up to 250%) of E2-induced ROS production in the neuroblastoma cell line SH-SY5Y (Figure 1B). To verify the ROS production results as measured by the DCFH-DA 96-well assay, we treated MCF7 (Figure 1C) with exogenous hydrogen peroxide. A significant increase in DCF fluorescence was detected by the 96-well DCFH-DA assay with hydrogen peroxide concentrations in the range of 1 nM to 500 μ M. These findings validate that intracellular ROS such as hydrogen peroxide can be detected in our cell model by

this assay. To confirm the production of ROS by E2, we pretreated MCF7 cells with the antioxidant NAC and the glutathione peroxidase mimic ebselen. A significant reduction in E2-induced ROS by both antioxidants, NAC and ebselen, is shown in Figure 1D. On the basis of these findings we conclude that E2 can stimulate a rapid production of intracellular ROS in breast cancer cells and neuroblastoma cells.

The Metabolic Pathway Responsible for E2-Induced ROS. To identify the source of intracellular ROS, we tried to suppress E2-induced ROS production using selective chemical blockers (Figure 2). Our results excluded the involvement of xanthine oxidase since allopurinol was ineffective in preventing ROS production; instead, we observed an increase in ROS production in the presence of allopurinol. NADPH oxidase was also excluded as a source because DPI could not prevent the E2-induced rise of ROS. E2-stimulated ROS production remained unaffected in the presence of the nitric oxide synthase (NOS) blocker L-NMMA; instead, we observed an increase in ROS production. Since DCFH can be oxidized by a variety of oxidants such as hydroxyl radical, superoxide anion, hydrogen peroxide, and nitric oxide, we used alternative assays to detect the presence of nitric oxide and superoxide anion. The nitric oxide sensitive fluorescent dye DAF-FM diacetate did not show a significant difference

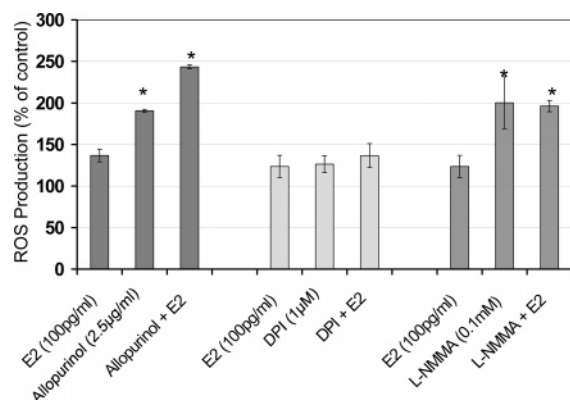


FIGURE 2: E2-induced ROS is not reduced by blockers of xanthine oxidase, NADPH oxidase, and NOS. MCF7 cells were pretreated for 1 h with allopurinol, DPI, and L-NMMA followed by E2 exposure for 15 min. Data from three independent experiments are presented as ROS production with controls set at 100% (\pm SD). Values that are significantly different from E2 treatment alone ($P < 0.05$) are indicated with an asterisk (*).

in the treatment groups (data not shown). Dihydroethidium was also used to detect superoxide anion; however, we did not observe a significant difference in the E2 treatment groups (data not shown). On the basis of these results, nitric oxide and superoxide anion were not considered to be the identity of the E2-induced oxidant. Given that the DCFH probe is selective for hydrogen peroxide and based on our results which show a reduction in E2-induced ROS production when treated with hydrogen peroxide specific antioxidants NAC and catalase (data not shown), the identity of the E2-induced oxidant appears to be hydrogen peroxide.

Since mitochondria are a major source of ROS in epithelial cells, we used several different approaches to test whether E2-induced ROS occurred via mitochondria. MCF7 cells were treated for 4 days with chloramphenicol, a mitochondrial protein translation blocker, which is known to disable mitochondrial function by preventing the synthesis of respiratory chain complexes. A significant decrease in E2-induced ROS production was observed in chloramphenicol-treated cells (Figure 3A). MCF7 cells were also treated with rotenone, a specific blocker of mitochondrial complex I. Rotenone treatment reduced E2-induced ROS production similar to that of chloramphenicol. To verify plate reader results of the role of mitochondria as a source of intracellular ROS, we used flow cytometry to measure ROS production using DCFH. MCF7 cells were pretreated with 1 μ M rotenone for 1 h followed by a 30 min E2 (100 pg/mL) treatment. DCF fluorescence was measured with a FACStar Becton Dickinson flow cytometer. As shown in Figure 3B, the representative histogram shows an overlay of three different treatments. A significant increase of E2-induced ROS production is represented by a shift in DCF fluorescence to the right of the control. Rotenone effectively suppressed E2-induced ROS as shown by a shift in DCF fluorescence to the left of the control. To further identify the source of intracellular ROS production, confocal microscopy was performed on E2-treated MCF7 cells with the fluorophores DCFH and Mitotracker Deep Red. In the confocal image of MCF7 cells shown in Figure 3C (ii), E2 treatment increased the intensity of DCF, which represents ROS production in the perinuclear region. In Figure 3C (iv), E2 treatment showed an increase in DCF fluorescence in dual-labeled

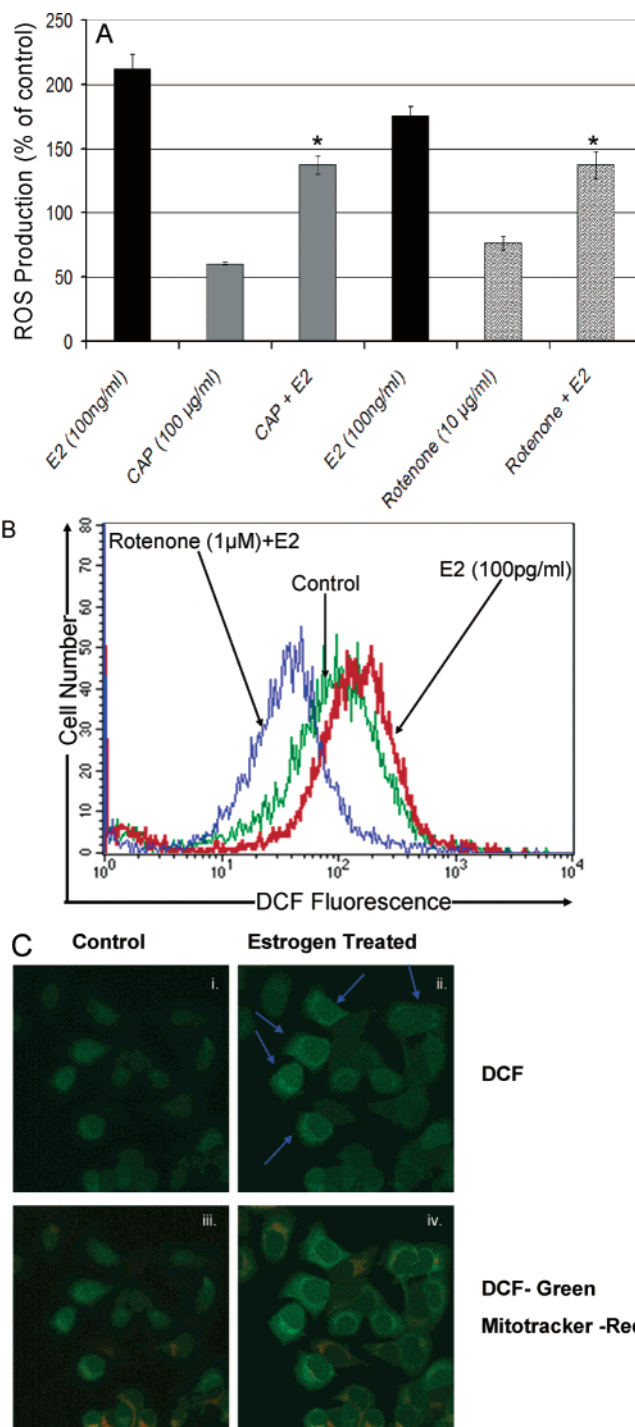


FIGURE 3: (A) E2-induced ROS is reduced by blockers of mitochondrial function. DCF assay on MCF7 cells treated with mitochondrial blockers. Cells were pretreated with chloramphenicol (CAP) for 4 days prior to E2 or pretreated with rotenone for 15 min. Data from three independent experiments are presented as ROS production with controls set at 100% (\pm SD). Values that are significantly different from estrogen treatment alone ($P < 0.05$) are indicated with an asterisk (*). (B) Rotenone reduces E2-induced ROS in breast cancer cells. Flow cytometry analysis of the effect of rotenone on E2-induced ROS. The histogram shown is an overlay of three different experiments. (C) Mitochondria and E2-induced ROS are colocalized at the perinuclear region. (i) Control MCF7 cells with DCF (green); (ii) E2 (100 pg/mL) treated MCF7 cells at 5 min with DCF. Cells with the intensely oxidized DCF are indicated by the blue arrows; (iii) control MCF7 cells dual labeled with DCF and Mitotracker Deep Red; (iv) E2 (100 pg/mL) treated MCF7 cells at 5 min dual labeled. Magnification = 63 \times (reproduced at 46% of original figure size).

(DCFH and Mitotracker Deep Red) MCF7 cells. The orange fluorescence represents colocalization of ROS and mitochondria. In some cells, a high DCF (in green) intensity overwhelmed the Mitotracker Deep Red.

Mechanism of E2-Stimulated Intracellular ROS Production. To determine whether E2-generated ROS production could be initiated at the level of the plasma membrane, MCF7 and T47D cells were treated with a cell membrane impermeable form of estrogen, E2-BSA (Steraloids, Inc.). E2-BSA (100 ng/mL) treatment increased ROS production by as much as 179% in both breast cancer cells (Figure 4A). The actin cytoskeleton was chosen as a potential mediator of E2-induced ROS production from the plasma membrane based on reports in fibroblast cells which show that reorganization of the actin cytoskeleton leads to the generation of ROS (14). To evaluate whether the actin cytoskeleton can control the production of ROS in epithelial cells, MCF7 cells were treated with cytochalasin D (Figure 4B). Cytochalasin D is a cell-permeable disruptor of actin microfilaments which is reported to reorganize the cytoskeleton leading to ROS production and served as a positive control (15). Upon the addition of cytochalasin D (0.05 μ M) ROS production increased 160% in MCF7 cells. Integrins were selected as a potential E2 binding site on the plasma membrane because they are reported to interact with the cytoskeleton and signal ROS production in fibroblast cells (14). To determine whether integrin ligation can stimulate ROS production in epithelial cells, adherent and suspended MCF7 cells were treated with various dilutions of anti- $\alpha 5\beta 1$ mAb. Anti- $\alpha 5\beta 1$ mAb has been reported to stimulate hydrogen peroxide production in fibroblast cells (14), and therefore it was used to test whether ligation of $\alpha 5\beta 1$ integrin produces ROS and if this process depended on cell adhesion. Integrin engagement with anti- $\alpha 5\beta 1$ mAb stimulated ROS production which was dependent on cell adhesion (Figure 4B). We also tested whether E2-induced ROS production required cell adhesion. An increase in ROS production was observed only in the adherent cells, which suggests that this process depends on cell adhesion, the cytoskeleton, and integrins (Figure 4C).

Since E2-induced ROS production depended on cell adhesion, we evaluated whether cdc42, a marker of cell adhesion, was activated at this early (30 min) time point. There was no significant change in the activated form of cdc42; however, the total protein level increased in response to E2 treatment (Figure 5A). Next, we evaluated whether markers of cell adhesion were modulated by E2 treatment at the early time point. To characterize whether new cell adhesion was upstream or downstream of the ROS source, we measured the activity of FAK and Pyk2. We did not detect a significant increase in the activated forms of FAK phosphotyrosine (pY)³⁹⁷ or pY⁸⁶¹ in Figure 5B. However, E2 treatment significantly increased Pyk2 pY⁴⁰² activation at 30 min (Figure 5B). To determine whether the phosphorylation of Pyk2 pY⁴⁰² is dependent on ROS generation, we treated MCF7 cells with the antioxidant NAC. The antioxidant NAC significantly inhibited Pyk2 pY⁴⁰² activation by both hydrogen peroxide and E2, which suggests that E2-induced cell adhesion depends on oxidants, and it is downstream of the ROS source (Figure 5B,C).

Recently, integrins were reported to engage mitochondrial ROS production through the activation of cytoskeletal protein Rac-1 in fibroblast cells (15). We measured the activation

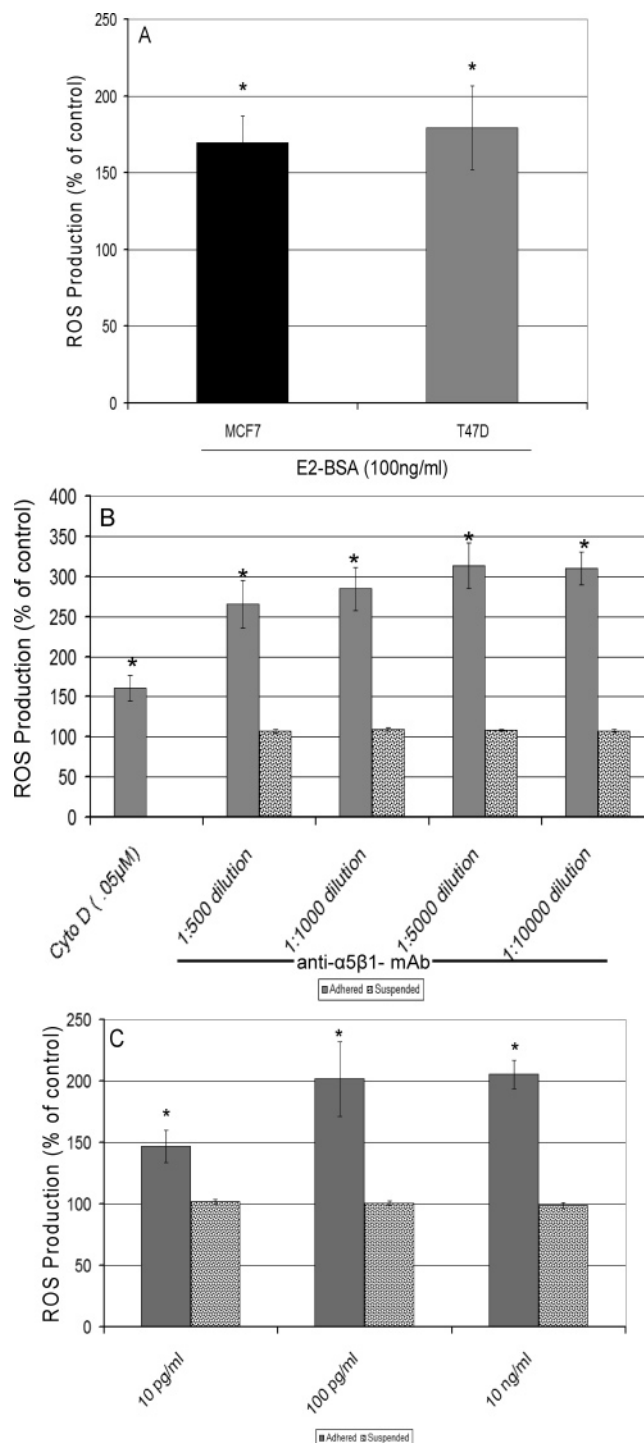


FIGURE 4: (A) E2-induced ROS is initiated at the plasma membrane. DCF assay on MCF7 and T47D breast cancer cells stimulated with 0.05% DMSO (control) and E2-BSA (100 ng/mL) for 15 min. (B) ROS production is mediated by the cytoskeleton and integrins in breast cancer epithelial cells. DCF assay on adherent and suspended MCF7 cells that were treated with anti- $\alpha 5\beta 1$ mAb for 15 min. Cytochalasin D treatment served as a positive control to indicate cytoskeletal-stimulated ROS production. (C) ROS formation in breast cancer epithelial cells depends on cell adhesion prior to E2 treatment. DCF assay of adherent and suspended MCF7 cells treated with E2 for 15 min. Data from three independent experiments are presented as ROS production with controls set at 100% (\pm SD). Values that are significantly different from controls ($P < 0.05$) are indicated with an asterisk (*).

of Rac-1 by E2 to evaluate whether ROS production may also occur by this pathway in our epithelial cell model. To

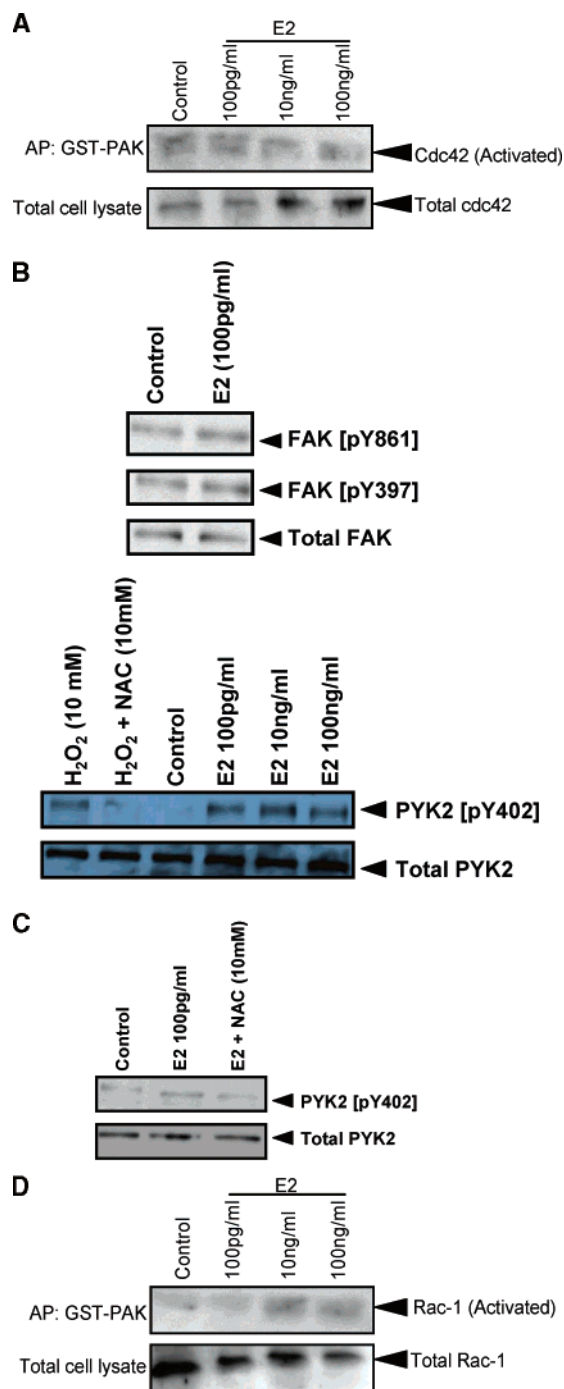


FIGURE 5: E2 increases the total protein level of cdc42. MCF7 cells were stimulated with E2 or vehicle alone (0.05% DMSO) for 30 min. Equal volumes of whole cell lysate were affinity precipitated (AP) with PAK-1 PBD bound to glutathione agarose beads. (A) Western blot analysis of affinity-precipitated cdc42 detected with anti-cdc42 mAb. (B) E2 activates the cell adhesion kinase Pyk2. Western blot analysis of FAK phosphotyrosine (pY) 861 and pY397 activation and of Pyk2 pY402 activation by E2 and/or H_2O_2 treatment. (C) NAC reduces E2-induced activation of Pyk2. Western blot analysis of Pyk2 activation by E2. (D) E2 rapidly activates cytoskeletal protein Rac-1. Western blot analysis of Rac-1 activation by E2. Precipitated Rac-1 was detected by IB with anti-Rac-1 mAb. The blots shown are representative of at least three separate experiments.

determine whether Rac-1 activation occurs by E2 treatment in MCF7 cells, we performed an affinity precipitation for Rac-1 using GST-PAK PBD on whole cell lysate. Proteins were separated 12% SDS-PAGE and blotted with anti-Rac-1

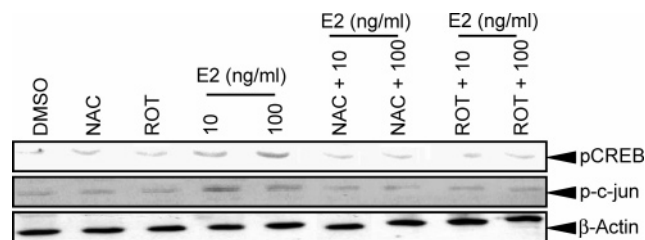


FIGURE 6: E2-induced phosphorylations of CREB and c-jun are reduced by the antioxidant NAC and rotenone. MCF7 cells were pretreated with 10 μ g/mL rotenone for 15 min or pretreated with NAC (10 mM) for 3 h followed by E2 stimulation for 3 h. Western blot was performed using specific mAbs for phospho-CREB (Ser-133) and c-jun to detect the protein expression in whole cell lysate.

mAb (Figure 5D). We observed a dose-dependent increase in Rac-1 activation after a 30 min treatment with E2. In addition, E2-induced Rac-1 activation was not prevented by cotreatment with the antioxidant NAC (data not shown), which suggests that Rac-1 is upstream of the ROS production.

E2-Induced ROS Signaling to Redox-Sensitive Transcription Factors. Oxidants are known to stimulate the phosphorylation of proteins c-jun and CREB; therefore, we tested whether E2-induced ROS modified the phosphorylation of these transcription factors. Phosphorylation of c-jun and CREB was increased by as much as 80% and 120%, respectively, by 3 h E2 treatment, and this phosphorylation was decreased to the level of control when cotreated with the antioxidant NAC (Figure 6). Since mitochondria are the likely source of E2-induced ROS, we also used the mitochondrial blocker rotenone to test whether c-jun and CREB phosphorylation depended on mitochondria. Rotenone treatment dramatically reduced the phosphorylation of c-jun and CREB to the control level (Figure 6). The data suggest that E2-induced phosphorylation of c-jun and CREB requires ROS, which may be of mitochondrial origin.

The TransAM AP-1 c-jun and pCREB assays were used to determine whether E2-induced ROS increased AP-1 binding to the TPA-response element (TRE) and pCREB binding to the cAMP-responsive element (CRE). The antioxidant NAC significantly decreased E2-induced phospho-c-jun binding (Figure 7A) while ebselen and rotenone had no effect (data not shown). Both antioxidants NAC and ebselen significantly decreased E2-induced pCREB binding to the level of the control (Figure 7B). The mitochondrial blocker rotenone also reduced pCREB binding to the control level. Since oxidants are reported to activate nuclear respiratory factor 1 (NRF-1), we performed an electrophoretic mobility shift assay (EMSA) to determine whether E2-induced ROS increased NRF-1 binding to its consensus sequence found in the human mt TFA promoter region. As shown in Figure 7C, ebselen significantly decreased E2-induced NRF-1 binding. On the basis of these results, E2-induced oxidants increase the transcriptional activity of redox-sensitive transcription factors AP-1, pCREB, and NRF-1.

DISCUSSION

The data presented here lead to major novel findings that physiological concentrations of E2 stimulate a rapid production of intracellular ROS and ROS formation in epithelial cells depends on cell adhesion, the cytoskeleton, and inte-

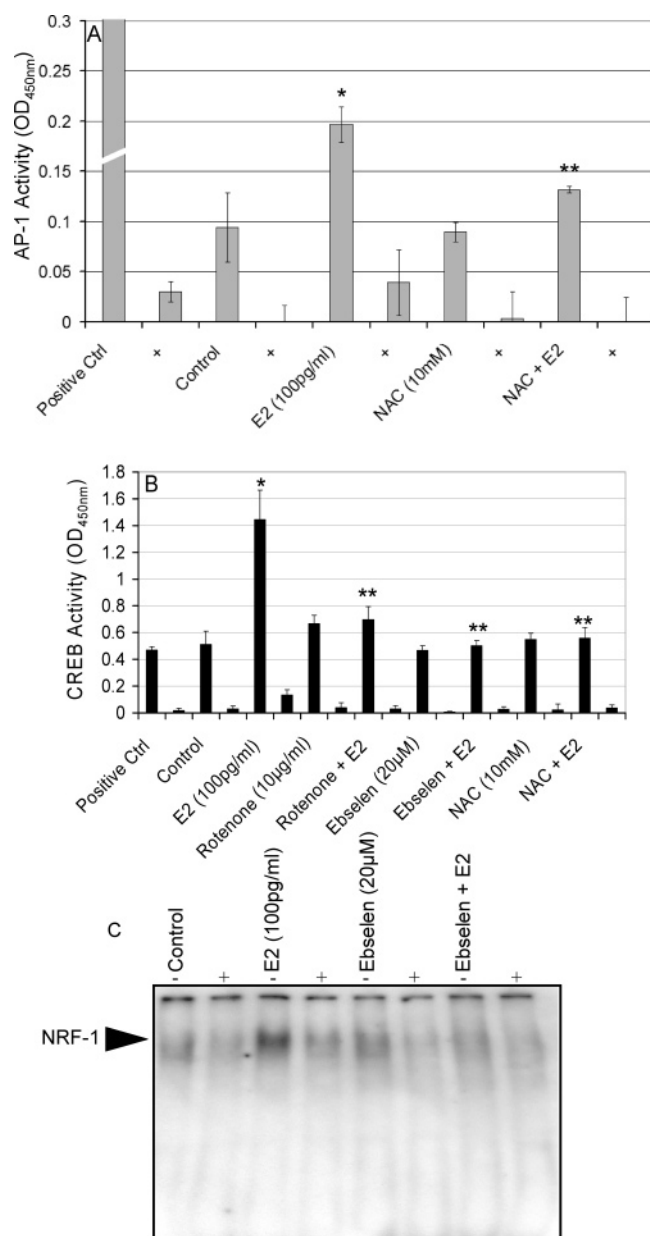


FIGURE 7: E2-induced AP-1 binding activity is reduced by NAC. MCF7 cells were pretreated with NAC for 2 h prior to E2 stimulation for another 3 h. (A) TransAM AP-1 assay on nuclear extracts. Positive control (OD_{450nm} = 0.7) is TPA-treated K-562 cell nuclear extract. The phospho-c-jun Ab recognizes phosphorylated Ser-73. (B) E2-induced CREB binding activity is reduced by antioxidants and rotenone. TransAM pCREB assay on nuclear extracts. Positive control is forskolin (2.5 μg) treated WI-38 nuclear extract. TransAM pCREB Ab recognizes Ser-133 phosphorylated CREB for the detection of activated CREB. (A, B) Data from three independent experiments are presented as mean OD_{450nm} ± SD. (+) competitor; (*) indicates treatment significantly different from control ($P < 0.05$), and (**) indicates treatment significantly different from E2 ($P < 0.05$). (C) E2-induced NRF-1 binding activity is reduced by ebselen. Ebselen was pretreated for 1.5 h followed by E2 treatment for 3 h. Nuclear extracts were collected, and 5 μg of extract was loaded for all samples. (–) No competitor; (+) with competitor. EMSA with NRF-1 consensus sequence from human mtTFA promoter region. The blot shown is representative of at least three experiments.

grins. These events occur earlier than ER-mediated genomic actions. E2-stimulated ROS production does not depend on the presence of the ER in breast cancer cells as the ER-negative cell line MDA-MB 468 produced ROS equal to that

of ER-positive cell lines MCF7, T47D, and ZR75.1. Interestingly, the neuroblastoma SH-SY5Y cells, not sensitive to estrogen-induced cell growth, showed a higher amount of E2-induced ROS production compared to ER-negative breast cancer MDA-MB-468 cells. Furthermore, chemical blockers such as NAC, ebselen, chloramphenicol, and rotenone which are not ER antagonists prevented E2-induced ROS-mediated effects. The finding of this study showing that ROS formation upon E2 exposure is not only able to explain oxidative damage to estrogen-dependent breast tumors and subsequent genetic alterations as reported earlier by Malins and others (16–18) but also provides mechanistic support to the generation of mutations by physiological concentrations of estrogens (19).

The extensive work on ERs indicates that they are involved in the promotion of estrogen-induced tumorigenesis, but their role is limited to the promotion of initiated cells. The critical sequence of genetic alterations involved in estrogen-induced carcinogenesis cannot be explained on the basis of ER-mediated pathways. The mechanisms by which estrogenic chemicals control the initiation and progression of cancer remain the subject of a long-standing controversy, in part because E2-induced DNA base modifications have been reported to occur at pharmacological and higher concentrations (20). Until now, the production of ROS by physiological concentrations of E2 has not been reported. Since ROS are known to modify DNA (20), our study supports the idea that ROS generated from physiological levels of E2 may lead to DNA modifications. Formation of estrogen–DNA adducts in mammary tissue, human breast tumor, and surrounding normal tissues has now been shown by combined LS-MS-MS and LC-nano ES tandem mass spectrometry (21). In addition, 4-hydroxycatechol estrogen (CE) conjugates with glutathione or its hydrolytic products (cysteine and NAC) have been detected in picomolar amounts in both tumors and hyperplastic mammary tissues from ER knockout/Wnt-1 mice, demonstrating the formation of CE-3,4-quinones (22). However, it is unlikely that these base modifications occur immediately after E2 exposure, as E2 would have to be hydroxylated and oxidized to a reactive intermediate in the endoplasmic reticulum prior to its transport to the nucleus. Although this type of adduction may play a role in the generation of mutations, it appears to be a later event. In our studies of E2-induced ROS generation in MCF7 and other cells, we were not able to find any hydroxylated estrogen metabolites or their adducts immediately after addition of E2.

The ligation of $\alpha 5 \beta 1$ integrins at the plasma membrane and reorganization of the actin cytoskeleton have been shown to mediate ROS production through the activation of Rac-1 in fibroblast cells (14). Whether E2-BSA binds to integrins or a plasma membrane ER is not known; however, we have shown that integrin ligation results in the production of ROS in our cell model, and therefore it is biologically plausible that the rapid E2-induced activation of cytoskeletal protein Rac-1 may also utilize a similar mechanism. In turn, activated Rac-1 may signal to the mitochondria via the cytoskeleton to generate oxidants. More specifically, mitochondrial tubulin and microtubule-associated proteins (MAPS) are reported to bind to porin or the voltage-dependent anion channel (VDAC), a component of the permeability transition pore (23). This association of the cytoskeleton with VDAC could

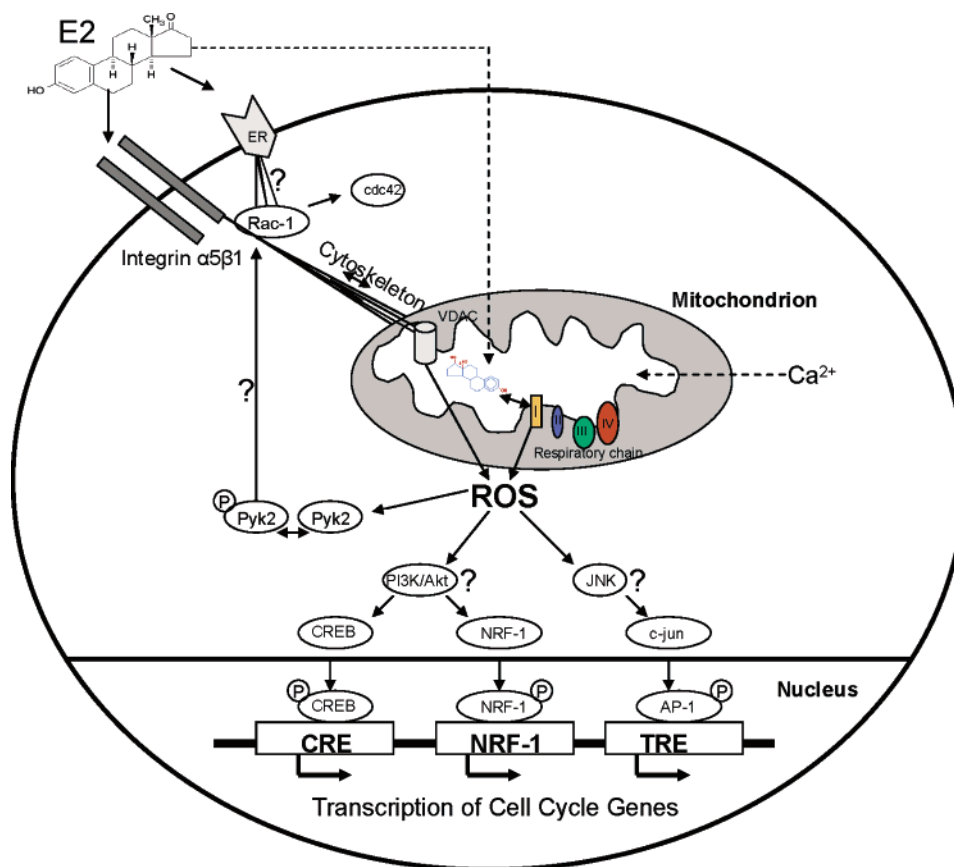


FIGURE 8: Schematic model outlining E2-induced ROS activation of transcription factors and Pyk2. E2 binding to integrin or a plasma membrane ER stimulates the reorganization of the cytoskeleton which activates the cytoskeletal protein Rac-1. Activated Rac-1 signals via the cytoskeleton to mitochondria where cytoskeletal interactions with VDAC increase the $\Delta\Psi_m$ and lead to an increase in ROS. E2 may also act directly at the level of the respiratory chain where it binds to complex I, resulting in a block of electron flow and generation of ROS. ROS increases the phosphorylation of Pyk2 leading to an increase in cell adhesion. ROS may also activate PI3K/Akt and JNK which in turn phosphorylate transcription factors NRF-1, CREB, and c-jun. The net result is binding of NRF-1, CREB, and AP-1 and increased transcription of cell cycle genes.

be biologically significant because the actin filament severing and capping protein gelsolin has been reported to modulate mitochondrial membrane potential ($\Delta\Psi_m$) by its interactions with VDAC (24). Therefore, Rac-1 may modulate VDAC activity via the cytoskeleton, leading to a rise in $\Delta\Psi_m$ and ROS formation.

ROS are now widely accepted to participate in signal transduction pathways, but the signaling pathway(s) activated by E2-induced ROS and the resulting changes in gene expression are not known. This led us to investigate the biological effects of E2-induced ROS at the protein level. Both oxidants and estrogen have been shown to phosphorylate transcription factors c-jun and CREB (25–27). In this study, we have shown that antioxidants NAC and/or ebselen reduced the phosphorylation of c-jun and CREB, which suggests that E2-induced ROS enhanced the phosphorylation of these redox-sensitive transcription factors. Since our objective was to investigate the effect of ROS on c-jun and CREB, the kinase(s) responsible for their phosphorylation is (are) not clear. We postulate that the kinase responsible for c-jun phosphorylation is the c-Jun-NH₂-terminal protein kinase (JNK) based on reports which demonstrate that E2 (10^{-9} M) can induce a rapid but sustained increase of JNK activation and that mitochondrial ROS can activate JNK (26, 28). We also postulate that PI3K/Akt is responsible for the phosphorylation of CREB given that ROS can signal CREB

via this kinase (25). However, the involvement of calcium–calmodulin kinase II (CaMKII) upstream of pCREB cannot be ruled out since E2 has been shown to activate pCREB via CaMKII (29) and our recent data showed that E2 increased the calcium concentration in a high calcium capacity mitochondrial population (30). Although NRF-1 phosphorylation was not measured, it is biologically plausible for ROS to signal the phosphorylation of NRF-1 based on a study which demonstrated ROS-induced activation of NRF-1 by PI3K/Akt (13). Besides the effect of ROS on the phosphorylation state of proteins, we have also shown that E2-induced oxidants increase the transcriptional activity or binding of AP-1, CREB, and NRF-1 to their respective DNA response elements. Since we have seen that E2-induced mitochondrial ROS control the expression of early G₁ phase genes (Feltz and Roy, unpublished results), these findings suggest that E2-induced ROS increase the transcriptional activity or binding of AP-1, CREB, and NRF-1 to their respective DNA response elements responsible for the expression of early cell cycle genes (Figure 8). Since the redox-sensitive transcription factors c-jun, CREB, and NRF-1 are known to regulate cell cycle genes (13, 31, 32), our study suggests that E2-induced ROS may play a role in cell cycle progression of estrogen-dependent cells.

Another important novel biological consequence observed in our investigation deals with the contribution of E2 to cell

adhesion as shown by the increase in the total level of cdc42, a marker of cell adhesion, and increase in Pyk2 activation. We have recently shown that mitochondrial ROS cause the selective activation of Pyk2 in cardiac myocytes which cause the sequential activation of the small GTP binding factors Rac-1 and cdc42 (26). In contrast, the suppression of E2-induced Pyk2 activation by the antioxidant NAC observed in this study suggests that Pyk2 activation occurs downstream of Rac-1 in MCF7 cells. On the basis of our findings, it appears that Pyk2 activation is a late event in the E2-induced ROS signal pathway which could help in the adhesion of serum-starved nonadherent cells. A recent study that showed ROS increased cell adhesion by the activation of focal adhesion kinases supports this idea (33).

Recently, antiinflammatory agents have been shown to reduce the risk of breast cancer (34). It has also been reported that intake of vitamins such as C and E lowers the risk of breast cancer (35). Our results not only shed light on a mechanistic explanation for these results but also provide a sound mechanistic basis for the application of these types of chemopreventive strategies against breast cancer. Our observations suggest that the use of reductants, such as NAC, ebselen, and vitamin C, and other antioxidants could prove to be a useful preventive agent to lower the hazards associated with excess estrogen exposure and the development of breast cancer.

In summary, the present study is the first to demonstrate that physiological concentrations of E2 stimulate a rapid production of intracellular ROS which lead to the phosphorylation of c-jun and CREB and increased activity of redox-sensitive transcription factors NRF-1, c-jun, and CREB known to be involved in the regulation of cell cycle genes. These newly identified mechanisms of estrogen action in breast cancer cells suggest new targets for developing antioxidant-based anticancer chemopreventive agents.

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