Functional Estrogen Receptors in the Mitochondria of Breast Cancer Cells

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Steroid hormones have been reported to indirectly impact mitochondrial functions, attributed to nuclear receptor-induced production of proteins that localize in this cytoplasmic organelle. Here we show high-affinity estrogen receptors in the mitochondria of MCF-7 breast cancer cells and endothelial cells, compatible with classical estrogen receptors ER α and ER β . We report that in MCF-7, estrogen inhibits UV radiation-induced cytochrome C release, the decrease of the mitochondrial membrane potential, and apoptotic cell death. UV stimulated the formation of mitochondrial reactive oxygen species (mROS), and mROS were essential to inducing mitochondrial events of cell death. mROS mediated the UV activation of c-jun N-terminal kinase (JNK), and protein kinase C (PKC) δ , underlying the subsequent translocation of Bax to the mitochondria where oligomerization was promoted. E2 (estradiol) inhibited all these events, directly acting in mitochondria to inhibit mROS by rapidly up-regulating manganese superoxide dismutase activity. We implicate novel functions of ER in the mitochondria of breast cancer that lead to the survival of the tumor cells.

INTRODUCTION

Estrogen promotes the development, proliferation, migration, and survival of target cells including breast cancer. The actions of estradiol (E2) have traditionally been thought to occur through binding nuclear steroid receptors (Jensen and Jacobson, 1962). Nuclear estrogen receptors (ER) transcribe genes whose protein products lead to the biological actions of the sex steroid. Most mitochondrial proteins are transcribed in the nucleus and E2 and nuclear ER regulate some relevant genes in this respect. However, recent work has supported the idea that a second pool of ER, localized to the plasma membrane, also importantly contributes to the actions of E2 (Levin, 2001).

Additionally, a large pool of ER have been described in the cytoplasm of various target cells, but the precise localization and functions are unclear. Recent data support the idea that some ER localize to the mitochondria of MCF-7 cells (Chen *et al.*, 2004). However, it is largely unknown whether these receptors participate in any actions of the steroid that impact the function of this organelle or the whole cell.

In breast cancer, abnormal proliferation and the increased survival of transformed cells are essential to the pathogenesis of the disease (Wang, 2001; Chen *et al.*, 2004). Tumor therapies for this malignancy are particularly effective when cell survival mechanisms are disrupted, inducing apoptotic

cell death (Yu *et al.*, 1998; Wang, 2001). Regarding apoptosis, a lynchpin event for many stimuli to enact this form of cell death is the release of cytochrome C from mitochondria into the cytoplasm, after the development of the mitochondrial transition pore. Cytochrome C in the cytoplasm complexes to and oligomerizes apoptosis activating factor-1 (Apaf-1), leading to the activation of caspase 9 and the effector caspase cascade. Effector caspases (such as caspases 3, 7, and 10) cleave and activate many substrates that commit the cell irrevocably to death (Wang, 2001). Translocation of cytochrome C to cytoplasm often precedes the decrease of the mitochondrial membrane potential (Danial and Korsmeyer, 2004), another marker of subsequent cell death. Modulation of these events determines cell fate but whether and how E2 regulates the intrinsic mitochondrial pathway of apoptosis in breast cancer is unknown.

Relevant to these considerations, we identify high-affinity mitochondrial ER compatible with classical ER α and ER β in several cell types. In breast cancer, E2/ER promotes the proliferation and survival of these malignant cells. We speculated that because mitochondria are an essential part of both the extrinsic and intrinsic pathways of programmed cell death (Wang, 2001), the mitochondrial ER pool might contribute through several mechanisms to cell survival. We therefore determined how radiation induces the key mitochondrial events of apoptosis and the role of mitochondrial ER to prevent many of these steps.

MATERIALS AND METHODS

Cell Culture and Plasmid Preparation

MCF-7 and bovine aortic or mouse brain capillary EC were isolated and cultured as previously described (Razandi *et al.*, 2000, 2004a). The targeting of the E domain of ER α to the mitochondria was accomplished as follows. The mouse ER α -E domain was subcloned by RT-PCR using the primers 5'-TTGGATCCGAACAGCCTGGCCTTGTC-3' and 5'-AAACCGGTGTGGGCGCATGTAGGC-3'. The PCR product was cloned using the TOPO TA 2.1

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Abbreviations used: ER, estrogen receptor; ERK, extracellular-regulated protein kinase; JNK, c-Jun N-terminal kinase; MnSOD, manganese superoxide dismutase; PKC, protein kinase C; ROS, reactive oxygen species.

cloning kit (Invitrogen, Carlsbad, CA), into BamHI and AgeI restriction sites on the pECFP-Mito vector (Clontech, Palo Alto, CA). The vector was confirmed by sequencing. Nuclear and membrane-targeted E domain construction and validation was previously described (Razandi *et al.*, 2003, 2004b).

Mitochondrial Isolation

Mitochondria were isolated by the method adapted from Trounce *et al.* (1996). Subconfluent MCF7 cells or BAEC cells were collected by gentle scraping and centrifuged at 1000 rpm for 5 min to pellet the cells. Cell pellets were washed once with phosphate-buffered saline (PBS) and twice with buffer A (0.25 M sucrose, 210 mM D-mannitol, pH 7.8). Final cell pellets were resuspended in buffer A containing 1 mM EDTA and DNase (prevent aggregation by released DNA) and protease inhibitor cocktail (Sigma, St. Louis, MO). Cells were homogenized using 15–20 strokes of the pestle of a tight-fitting Dounce homogenizer until ~90% of the cells were broken. Homogenates were centrifuged at 1000 × *g* for 10 min to pellet the nuclear fraction. The supernatants were layered onto a discontinuous sucrose gradient (1.0–2.5 M) made up in buffer containing 10 mM Tris, pH 7.6, 2 mM EDTA, 2 mM DTT, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride. The mixtures were centrifuged at 2000 rpm for 30 min at 4°C producing a top layer (cytosol) and a mitochondrial layer at the 1.0–1.5 M sucrose gradient interface. The mitochondrial fractions were removed from the centrifuge tube with a 22-gauge needle and 1-ml syringe, washed in four volumes of mannitol/sucrose buffer, and centrifuged at 1000 rpm for 15 min at 4°C. The pellets containing mitochondria were suspended in PBS, pH 7.4, sampled for protein concentration (BCA method), and stored at –80°C until further analysis. Mitochondrial protein was determined by the method of Lowry.

Saturation Binding Studies

Saturation and competitive binding of ³H-estradiol were carried out on whole cells as described (Razandi *et al.*, 2003), and the cells then were fractionated into plasma membrane, nuclear (Razandi *et al.*, 2004b), and mitochondrial isolates by sucrose gradient centrifugation. Unlabeled E2 from 0.01 to 1000 nM was used for competition, and the studies were carried out at 60 min at 37°C. Free [³H]17-β-E2 was separated by resin adsorption of the ligand-receptor complex using the hydroxylapatite (HAP) technique (Weckslar and Norman, 1999). Binding was analyzed by Scatchard analysis, using the LI-GAND binding computer program. Purity of the subcellular isolates was established by Western blot of fractional proteins after SDS-PAGE separation and transfer to nitrocellulose. Antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA) and detection was accomplished using the ECL kit (Amersham, Indianapolis, IN).

ER Detection in Mitochondria

MCF-7 cells were cultured on coverslips overnight and then incubated for 20 min with Mitotracker dye (red; Molecular Probes, Eugene, OR) that localizes to the mitochondria. After washing with PBS, the cells were fixed with paraformaldehyde and permeabilized with 0.2% Triton-X and then incubated with antibody to either the C-terminus of ERα (Santa Cruz Biotechnology) or the N-terminus of ERβ (Zymed, South San Francisco, CA; Razandi *et al.*, 2004a). This was followed by second antibody conjugated to FITC (green color). ER antibodies preabsorbed with purified ERα or ERβ protein, or nonspecific IgG were used as additional negative controls. Western blot of specific proteins in isolated mitochondria, plasma membrane, and nuclear cell fractions was also performed, and samples from each condition were normalized by measuring the total protein (Bradford assay). Antibodies were from Santa Cruz Biotechnology.

Manganese Superoxide Dismutase Assay

Superoxide dismutase (SOD) activities were determined from protein-normalized aliquots from each condition, using a Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI). Tetrazolium salt was used for detection of superoxide radicals, and the radicals generated by provided xanthine oxidase were quenched by known amounts of exogenous SOD. This generated a standard curve for comparison to cell samples containing endogenous SOD activity. MCF-7 cells were collected by scraping and centrifuged, and the pellets were sonicated in cold buffer containing sucrose. In some experiments, a mitochondrial extract was prepared. The cell or mitochondrial extract supernatant was centrifuged at 1500 × *g* for 5 min at 4°C and then centrifuged at 10,000 × *g* for 15 min at 4°C. The resulting supernatant contained cytosolic SOD (CuZnSOD), and the pellet contained mitochondrial SOD (MnSOD). Also, in whole cell preparations, we used 2 mM potassium cyanide to inhibit both Cu/Zn-SOD and extracellular SOD, resulting in the detection of only MnSOD activity. The samples were analyzed using a plate reader with a 450-nm filter. Each data point was performed in triplicate, and the results from multiple experiments were combined and reported as mean absorption ± SE.

Mitochondrial Functions in Apoptosis

MCF-7 cells were exposed to 50 Joules of UV irradiation in 1 s to induce apoptosis (Razandi *et al.*, 2000, 2004a). The cells were processed for Western

blotting of cytochrome C, both released into cytosol and remaining in mitochondria over a 4-h period. Some cells were exposed to 10 nM E2 just before irradiation and for the duration of the experiment. In additional experiments, HCC-1569 were transiently transfected to express the E domain of ERα, incorporated into plasmids that included cell membrane, mitochondrial, or nuclear targeting sequences (Clontech), the cells were recovered overnight, and similar studies were carried out.

Decreases in the mitochondrial membrane potential were evaluated using the Apo Alert Mitochondrial Membrane Sensor Kit (Clontech Laboratories). MCF-7 cells cultured on coverslip were exposed to UV ± 10 nM E2 with or without ICI182780 and then left for 4 h. Cells were also exposed to ICI alone or E2 alone as controls. When using HCC-1569 cells, the cells were grown on coated glass bottom culture dishes (MatTek, Ashland, MA). After transient transfection with the E domain of ERα targeted to different compartments, cells were synchronized and treated as described. At the end of the treatment, all cells were washed in PBS and incubated with Mitosensor dye (5 μg/ml) for 20 min at 37°C. The cells were examined at 20× by fluorescent confocal microscopy using a band-pass filter. The Mitosensor dye aggregates in the mitochondria of healthy cells and shows red fluorescence. In apoptotic cells, the mitochondrial membrane potential is altered; Mitosensor cannot accumulate in the mitochondria and remains as a green fluorescent monomer in the cytoplasm.

ROS Formation

Cultured cells were loaded with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes) for 1 h before the apoptotic stress. In cells, esterases cleave the acetate esters to release free CM-H2DCF, which is nonfluorescent. After oxidation by ROS, CM-H2DCF is converted to green-fluorescing dichlorofluorescein (DCF; Curtin *et al.*, 2002). After brief UV exposure, the cells were left for 4 h, then washed, fixed, and permeabilized with PBS containing 20% ethanol and 0.1% Tween 20. In some experiments, 10 nM E2 was added for 4 h. Cell extracts were centrifuged, and supernatants collected. DCF fluorescence was quantified with a fluorescence plate reader using 450-nm excitation and 530-nm emission filters. Confocal microscopy provided a qualitative assessment. The study was carried out three times, and the mean and SD were analyzed by ANOVA plus Scheffé's test at a *p* < 0.05 level of significance.

Bax Dimerization and Translocation

MCF7 cells at near confluency were synchronized and then subjected to UV irradiation ± E2 for 6 h except control cultures (sham irradiated). Isolated mitochondria were washed, and the cross-linker, dithiobis (DSP; Pierce, Rockford, IL), was added in dimethyl sulfoxide. Cross-linking for 30 min at room temperature preceded centrifugation at 10,000 × *g* for 15 min, and the mitochondria pellet was suspended in lysis buffer. Anti-Bax monoclonal antibody (6A7, Sigma) was preincubated with protein A Sepharose. The cell lysates were normalized for protein content, and 500 μg of total protein in Chaps-containing lysis buffer was added to the tube containing Bax antibody-loaded beads and incubated at 4°C overnight. After rinsing, beads were collected and Bax protein was eluted with sample buffer for Western blotting. The separated proteins were transferred to nitrocellulose followed by blocking with 5% (wt/vol) nonfat milk powder in buffer. Membranes were probed with primary antibody followed by peroxidase-conjugated secondary antibody, visualized using an ECL detection kit.

Interfering RNA to PKC and MnSOD Studies

Double-stranded RNA for PKCδ, PKCε, PKCβ (control), or MnSOD or GFP (control) was obtained from Santa Cruz Biotechnology (SC-36521, 36252, 29450) or Dharmacon (Boulder, CO; M-009784). We transfected 2.5 μg each of the siRNAs in MCF-7 using OLIGOfectamine as described (Razandi *et al.*, 2004b), recovered and synchronized the cells over 48 h, and then carried out the various experiments. Immunoblots for the proteins were carried out 48 h after transfection to validate the protein knockdown and specificity of the constructs.

PKC and JNK Activity

PKCδ and PKCε activities were determined as phosphorylation of the protein isoform at the active site. Cells were exposed to various conditions and the cells were lysed, then separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to tyrosine 311 (PKCδ) or serine 729 (PKCε; Santa Cruz) or with antibodies to determine total PKC protein. JNK activity was determined as previously described (Razandi *et al.*, 2000). SP600125 (JNK1 inhibitor) and Rottlerin (PKCδ inhibitor) were from Calbiochem (San Diego, CA). PKC isoform activity against peptide substrate was also determined. Cells were incubated with 10 nM E2 in the presence of absence of UV exposure for 4 h. PKCδ or PKCε was immunoprecipitated from MCF-7 cells lysate using specific antibodies (Santa Cruz), and the proteins were normalized for an in vitro activity assay. The tube activity assay included ³²P-ATP, substrate peptide (glycogen synthase kinase; Calbiochem), and normalized PKC isoform protein from each experimental condition. PKC activity was determined after a 30-min incubation at 30°C, and the phosphorylated substrate was identified after SDS-PAGE. Total PKC protein was determined by Western blot for sample normalization.

RESULTS

Identification of Mitochondrial ER

We first established high-affinity, saturable ER in plasma membrane, nucleus, and mitochondrial cell fractions of MCF-7 cells ($K_d = 0.2\text{--}0.3$ nM; Figure 1A, left). Approximately 85% of receptors were present in the nucleus, 5% in the plasma membrane, and 10% in the mitochondria. The lack of contamination of cell fractions was demonstrated by Western blot for integral membrane (5'nucleotidase [NT]), nuclear (transportin and NTF2), and mitochondrial proteins (cytochrome C; Figure 1A, right). High-affinity mitochondrial ER were also found in endothelial cells (EC; unpublished data).

Using antibodies to traditional ER isoforms, we determined in MCF-7 cells that endogenous ER α and ER β are present in plasma membrane, nucleus, and mitochondrial cell fractions. The overall cellular ER β protein is considerably less abundant than ER α (Figure 1B, left), consistent in that ER β is produced in low abundance in breast cancer (Fuqua and Cui, 2004). Interestingly, the small amount of ER β is concentrated in mitochondria. This is different from EC, where ER β (and ER α) are predominantly expressed in the nucleus, and the relative amount of ER α exceeds ER β in mitochondria (Figure 1B, right). Confocal microscopy confirmed ER α in membrane, nuclear, and mitochondrial cell compartments of the MCF-7 cell (Figure 1C, left). ER β was also detected (Figure 1C, right), and both ER isoforms colocalized with the mitochondria specific dye, Mitotracker. Again, ER β was predominantly found in the mitochondria of MCF-7 (Figure 1C, right). Antibodies preabsorbed with purified ER α or ER β protein or nonspecific IgG showed no staining of proteins in the cells (unpublished data).

To further support the idea that mitochondrial ER α and ER β derive from the same genes coding for classical nuclear ER isoforms, we examined EC from combined ER α /ER β knockout mice (DERKO) (11). Wild-type EC demonstrated endogenous ER α in several cell locations, most prominently in the nucleus, and ER β was found substantially in mitochondria. In contrast, DERKO EC produced no detectable ER in any region of the cells (Figure 1D). Thus, in EC, mitochondrial ER derive from the same genes (ER α or ER β) that also produce nuclear and membrane ER (Razandi *et al.*, 2004b).

ER Functions in the Cell Survival of Breast Cancer

Breast cancer cells respond to irradiation by undergoing apoptotic cell death (Xia and Powell, 2002). We previously reported that E2 inhibits radiation-induced cell death in MCF-7 cells (Razandi *et al.*, 2000, 2004a). In part, this resulted from membrane-initiated steroid signaling to activation of ERK and PI3 kinases, perhaps impacting the mitochondria. E2/ER inhibition of JNK activity through undetermined ER pools or mechanisms also contributed (Razandi *et al.*, 2000, 2004a). Nuclear and membrane ER also signal to the up-regulation of genes such as Bcl2 (Dong *et al.*, 1999), the protein products of which promote long-term cell survival. Whether E2 acts directly at mitochondrial ER to impact cell fate is unknown.

To understand whether E2 influences apoptotic events in the mitochondria, we determined the release of cytochrome C from this organelle in UV-irradiated MCF-7 cells. UV induced the maximal translocation of cytochrome C from mitochondria into the cytoplasm at 60 min, significantly prevented by E2 (Figures 2A, left and right). Cytochrome C redistribution was also visualized in the intact cell, and ICI182780, an estrogen receptor antagonist, substantially although incompletely blocked the action of E2 (Figure 2B).

Irradiation of MCF-7 cells also decreased the mitochondrial membrane potential, reflecting the commitment to apoptosis (Figure 2C). The shift from mainly red to green color occurred as the dye failed to be taken up in the mitochondria of dying cells. E2 significantly decreased the numbers of cells showing loss of membrane potential by ~60%, substantially prevented by ICI182780.

Role of Mitochondrial ER

To determine whether E2 acted at mitochondrial ER, we targeted the ligand-binding domain (E domain) of ER α to the plasma membrane or nucleus (exclusive targeting to these pools shown in Razandi *et al.* 2004b), or to the mitochondria of ER negative, HCC-1569 breast cancer or CHO cells. Expression of the E domain in mitochondria was demonstrated by substantial overlap with Mitotracker dye and by Western blot of cell fractions (Figure 3A). We then examined cytochrome C translocation.

In HCC-1569 cells containing the mitochondrial-targeted E domain, E2 prevented the cytochrome C effects of UV (Figure 3B). UV also reduced the mitochondrial membrane potential in all cells, but this was significantly reversed by sex steroid in mitochondrial ER-targeted cells (Figure 3C). In ER null breast cancer cells expressing the pcDNA3 plasmid (strict control), there were no E2 effects (unpublished data), supporting the need for ER to mediate E2 actions. Interestingly, plasma membrane targeting of the ER α E domain also supported E2 reversal of both UV effects (Figure 3, B and C). Perhaps this resulted from ERK or PI3 kinase signaling from the membrane to cell survival (Razandi *et al.*, 2000, 2004a).

By contrast, targeted expression of the E domain in the nucleus did not significantly impact cytochrome C translocation or mitochondrial membrane potential, determined 1 and 4 h, respectively, after UV exposure. This nuclear only model fully supports E2-induced proliferation of breast cancer cells (Razandi *et al.*, 2004b), and a comparably truncated ER α supports nuclear transcription (Lopez *et al.*, 1999). We also targeted a full-length ER α to the nucleus and found a similar failure of E2 to reverse these events of apoptosis (unpublished data).

As a second model, we isolated mitochondria from MCF-7 cells, the purity established as in Figure 1A. UV stress induced the release of cytochrome C from mitochondria into the incubation medium (Figure 3D). E2 prevented this (lane 3), indicating direct action at this cytoplasmic organelle. To determine which ER isoform mediates this action, mitochondria were exposed to UV in the presence of 10 nM E2, PPT (an ER α agonist), or DPN (an ER β agonist; Harrington *et al.*, 2003). Each inhibited cytochrome C release, but the ER β agonist was more potent than the ER α agonist. Taken together, the combined effects of ER α and ER β agonists were roughly equivalent to the inhibition by E2, likely reflecting contributions from each mitochondrial ER isoform to steroid action.

Mechanisms of ER Action in Mitochondria

How does mitochondrial ER protect the viability of irradiated cells? In cancer, reactive oxygen species (ROS) are important to apoptosis induced by radiation, chemotherapeutic agents, and many cell stressors (Benhar *et al.*, 2002). In most stress circumstances, mitochondria generate the great majority of ROS (Finkel and Holbrook, 2000). ROS generation here is normally a result of oxidative phosphorylation, and several of the respiratory complexes produce ROS as a result of electron transport between complexes (Curtin *et al.*, 2002). Complexes I and III (especially the latter) have been particularly implicated during stress-related ROS formation.

Figure 1. High-affinity ER in mitochondria (A). Left, competition binding of [³H]17 β -E2 to nuclear, mitochondrial, and cell membrane fractions of MCF-7 cells. K_{dS} for nuclear, mitochondrial, and cell membrane receptors were 0.283, 0.290, and 0.287 nM, respectively, calculated by Scatchard analysis using the LIGAND computer program. The studies were repeated twice. Right, purity of cell fractions. Immunoblots of membrane protein (5'NT), nuclear proteins (transportin and NTF), Golgi protein (β -COP) and mitochondrial protein (cytochrome C) were carried out in the MCF-7 cell fractions. (B) Western blot of MCF-7 (left) or bovine aortic EC (right) cell fractions. Cell lysate was immunoprecipitated with specific antibodies

Figure 2. Mitochondrial effects of E2. (A) Left, cells were treated with brief UV exposure (50 J/s) and abundance of cytochrome C was determined in MCF-7 cell fractions by Western blot for up to 240 min. The study was repeated. Right, E2 prevents UV-induced cytochrome C release into the cytosol at 60 min. MCF-7 were briefly exposed to UV in the presence or absence of E2, and steroid was continued for 60 min. (B) A predominantly mitochondrial distribution of cytochrome c (control) changed to a largely diffuse cytosolic pattern in response to UV, prevented by E2. MCF-7 were briefly exposed to UV, with or without 10 nM E2, or E2+ICI182780 (1 μ M; ER antagonist) for 60 min. After fixing and permeabilizing the cells, incubation with the first antibody to cytochrome C was followed by second antibody conjugated to FITC. The study was repeated. (C) MCF-7 were briefly exposed to UV, the cells incubated with or without E2 or ICI182780 for 4 h, and then loss of membrane potential was determined using the Apo-Alert kit (Clontech) as described in *Materials and Methods*. Decreased mitochondrial membrane potential was seen as a color change from predominantly red to green. As controls, cells were also exposed to ICI or E2 alone without significant effect (unpublished data).

We first determined that UV induced a strong generation of ROS, substantially reversed by *N*-acetylcysteine (NAC), a nonspecific inhibitor of ROS generated at multiple sites in the cell. Inhibition of ROS generation also occurred in the presence of Rotenone, a specific inhibitor of the mitochondrial complex I, and potently by Mito-Q (Figure 4A). Rotenone either enhances (Ohnishi *et al.*, 2005) or inhibits (Chapman *et al.*, 2005; Sato *et al.*, 2005) ROS formation, depending on the insult and coupled state of mitochondria. Here, this compound inhibits ROS formation. The Mito-Q compound is a mitochondrial-targeted derivative of ubiquinone. By accepting electrons from complexes I and II, the reduced product, ubiquinol, strongly reduces ROS formation, influencing complex III function as well (Kelso *et al.*, 2001; James *et al.*, 2005). Mito-Q potently prevented ROS formation by UV, and E2 substantially prevented UV-induced ROS in MCF-7 cells, reversed by the ER antagonist, ICI182780 (Figure 4B).

E2 dose-responsively acted as a survival factor for MCF-7 cells, with significant effects seen at 1 nM E2 (Figure 4C). In contrast, 10 nM 17- α -E2, progesterone, or testosterone did not block UV-induced cell death, indicating steroid specificity. ER α - and ER β -specific agonists each significantly prevented UV-induced cell viability, with the ER β agonist being slightly more potent (Figure 4C). We also determined that NAC, rotenone, and especially Mito-Q prevented UV-induced loss of cell viability (Figure 4D). These results focus attention on mitochondrial ROS as regulated by UV and E2/ER to explain cell death and survival functions, respectively.

Figure 1 (cont). to the ER α and ER β isoforms, normalized for protein, and proteins were separated by gel electrophoresis, transferred, and then immunoblotted. Molecular weight markers are shown. (C) Immunofluorescence confocal microscopy of ER α (left) and ER β (right) in MCF-7. The cells were cultured on coverslips and then incubated for 20 min with Mitotracker dye (red; Molecular Probes). After washing, the cells were fixed and permeabilized and then incubated with antibody to either ER isoform, followed by second antibody conjugated to FITC (green color). Overlap is seen in yellow. (D) Lack of mitochondrial ER in ER α /ER β deleted cells. EC were isolated from the capillaries of mice bred for combined ER α and ER β deletion (DERKO). Confocal microscopy fails to show any ER in any cell compartment in EC from DERKO mice, whereas EC from wild-type mice show receptors for each ER isoform in various cell locations.

If mitochondrial ROS generation underlies the radiation-induced mitochondrial death program, then preventing oxidant formation should rescue these events. We found that UV-induced cytochrome C translocation was almost completely reversed by NAC, rotenone, and especially Mito-Q (Figure 4E). Additionally, Mito-Q prevented the loss of membrane potential (unpublished data). Thus, local ROS stimulation by UV and inhibition by E2 underlies mitochondrial cell fate events.

How might E2 prevent ROS formation and does this stem from mitochondrial ER? The dominant mitochondria-specific enzyme that reduces ROS formation in this organelle is MnSOD (Halliwell, 1999; Huang *et al.*, 2000). MnSOD rapidly reduces superoxide, the main mitochondrial ROS. We exposed whole cells or isolated mitochondria from MCF-7 to UV $\pm$ E2 and assayed for MnSOD activity. E2 strongly up-regulated MnSOD activity in MCF-7 cells, and UV partially prevented this action of the steroid (Figure 5A). Critically, isolated mitochondria responded to E2 with robust activation of MnSOD. We also determined the rapidity of E2 up-regulation of MnSOD activity; We found that E2 significantly stimulated enzymatic activity in the cells between 10- and 20-min exposure (Figure 5B). The stimulation was ER mediated, because ICI182780 prevented this action of E2 both at 20 min (Figure 5C, left) and at 4 h (right). We also found that mitochondrial MnSOD protein did not significantly vary over this time under any condition (Figure 5C, right). The rapid up-regulation of MnSOD activity coincides with the kinetics of kinase modulation by UV and E2. Interfering RNA that specifically knocked down MnSOD protein substantially reversed the ability of E2 to prevent ROS generation (Figure 5D). Thus, E2 rapidly prevents ROS formation mainly through the exclusively mitochondrial SOD. However, cross-talk from other ER pools to the mitochondrial MnSOD enzyme might contribute to this effect in the intact cell.

ROS Signals to JNK and PKC δ Activation

E2 prevents UV radiation and taxol chemotherapy from inducing apoptotic breast cancer cell death in part by inhibiting JNK activity through undetermined mechanisms (Razandi *et al.*, 2000). Here we found that UV-induced activation of JNK was substantially inhibited by

Figure 3. E domain of ER α mediates E2 effects at the mitochondria. (A) Left, expression of the mitochondrial-targeted E domain of ER α . CHO cells were transfected to express the E domain of ER α (green) targeted to the mitochondria, using a targeting vector containing a mitochondrial localization sequence (Clontech). Mitotracker dye indicates the mitochondria (red). The merged panel shows virtually complete colocalization of ER with Mitotracker dye (orange-yellow). Right, Western blot of ER α E domain targeted to the mitochondria and expressed in HCC-1569 cells. Western blot of cell fractions was accomplished with an antibody to the ER α ligand-binding domain (H222). (B) The E domain of ER α was targeted to the plasma membrane, mitochondria, or nucleus in HCC-1569 cells. Western blot of cytochrome C in cytoplasm at 60 min post-UV in the presence or absence of 10 nM E2 treatment is shown. The study is representative of two experiments. (C) Mitochondrial membrane potential and apoptosis in E domain-targeted HCC-1569 cells. Cells were briefly exposed to UV, then $\pm$ E2, or E2 alone, and membrane potential/apoptosis was determined at 4 h. Control cells were transfected but sham exposed to UV. The bar graph represents the mean plus SEM from three combined studies, 200 cells counted per condition in each. * $p < 0.05$ for control or E2 alone versus UV, + $p < 0.05$ for UV+E2 versus UV by ANOVA plus Scheffé's test. (D) ER α and ER β mediate E2 action at the mitochondria. Isolated mitochondria from MCF-7 cells were exposed to UV $\pm$ 10 nM of E2, PPT, or DPN, and cytochrome C release into the incubation medium or in the mitochondrial pellet was determined by Western blot at 60 min. The representative study was repeated twice additionally.

Figure 4. Reactive oxygen species (ROS) underlies cell fate decisions. (A) MCF-7 cells were exposed to UV $\pm$ antioxidants NAC (*N*-acetylcystine) 5 mM, rotenone 2.5 μ M, or Mito-Q 10 μ M. ROS production was determined 4 h after UV exposure by confocal microscopy using the fluorescent indicator, CM-H2DCFDA, and quantified by spectrofluorometry. The bar graph represents three studies combined. * $p < 0.05$ for control versus UV, + $p < 0.05$ for UV versus UV plus antioxidant. None of the antioxidant compounds had significant effects on ROS formation by themselves (unpublished data). (B) Estradiol/ER prevents UV-induced ROS formation. The cells were exposed to brief UV, with or without E2 or 1 μ M ICI182780, and incubated for 4 h. The bar graph is data from three combined experiments. * $p < 0.05$ for control versus UV, + $p < 0.05$ for UV versus UV+E2, ++ $p < 0.05$ for UV+E2 versus same plus ICI182780. (C) MCF-7 were exposed to UV $\pm$ 0.1–10 nM 17- β -E2, 10 nM 17- α -E2, 10 nM progesterone (P) or testosterone (T), or 10 nM PPT or DPN. Steroid compounds were continued for the length of the experiment. Cell viability was determined by the MTT assay at 4 h. Decreased viability is shown by a loss of spectral absorbance to the dye. Data are from three experiments; * $p < 0.05$ for control versus UV, + $p < 0.05$ for UV versus UV plus steroid. (D) MCF-7 were exposed to UV $\pm$ antioxidants and cell viability was determined by the MTT assay at 4 h. Data are from three experiments; * $p < 0.05$ for control versus UV, + $p < 0.05$ for UV versus UV plus antioxidant. (E) Inhibitors of ROS generation reverse UV-induced cytochrome C release into the cytoplasm. A representative study shown here was repeated. Actin serves as loading control.

Figure 5. Estrogen upregulates MnSOD activity to inhibit ROS. (A) Isolated mitochondria or whole MCF-7 cells were exposed to UV $\pm$ E2, or E2 alone for 4 h, and MnSOD activity was determined using a kit (Cayman). Control is sham UV-irradiated cells. The data are from three combined experiments, * $p < 0.05$ for control versus E2, + $p < 0.05$ for E2 versus E2 + UV. (B) Time course of MnSOD activity. MCF-7 cells were exposed to UV $\pm$ E2 and activity from discrete wells of cells was determined every 10 min for 1 h. Each data point is the mean of quintuplicate replicates, the

Figure 6. Role of JNK in UV-induced cell death. (A) MCF-7 cells were exposed to UV in the presence or absence of Mito-Q, and JNK activity was determined sequentially over a 4-h period. The top panel shows the 30 min time point. A representative study is shown, repeated once. (B) Cells were briefly exposed to UV $\pm$ SP 600125, a JNK-1 inhibitor, the latter continued for 4 h. Cytochrome C in the cytoplasm was determined by Western blot, and the study was repeated.

all the antioxidants, especially Mito-Q (Figure 6A). The results implicate mitochondrial ROS as the critical regulator of UV-induced JNK activation and suggest that E2 inhibition of ROS shown here underlies the inhibition of kinase activity (Razandi *et al.*, 2000). We then asked whether JNK activation

contributes to mitochondrial apoptosis events. A specific inhibitor of JNK1 activity, SP 600125, partially prevented UV-induced cytochrome C release into cytoplasm (Figure 6B) and also significantly blocked UV-induced loss of mitochondrial membrane potential (unpublished data).

Another important mediator of apoptosis in many cell insult models is the novel PKC isoform, PKC δ (Steinberg, 2004). This isoform translocates to mitochondria in response to H₂O₂, where it participates in the mitochondrial program of apoptosis (Majumder *et al.*, 2001). By comparison, PKC ϵ in some cell types contributes to cell survival (McJilton *et al.*, 2003; Murriel and Mochly-Rosen, 2003). We determined whether these PKC isoforms contributed to cell fate in MCF-7. After 30 min and persisting for 4 h, UV strongly activated PKC δ , noted as tyrosine 311 phosphorylation (Figure 7A). E2 strongly inhibited this activation. Furthermore, UV-activated PKC δ was significantly dependent on ROS (Figure 7B). We also determined PKC δ activity, using a PKC substrate peptide (from glycogen synthase kinase; Figure 7C). We found that UV stimulated, whereas E2 prevented PKC δ activation by UV. In contrast, PKC ϵ phosphorylation and activity were stimulated at 4 h by E2 but not by UV (Figure 7, A and C). Therefore, the two PKC isoforms could be differential mediators of cell fate in our system.

To support this, we investigated PKC functions in the modulation of cytochrome C release. To do this, we first validated that specific siRNAs for PKC δ and PKC ϵ knock down the intended target proteins (Figure 7D). Importantly, the PKC control siRNA to PKC β had no effects on either the δ or ϵ isoforms. Silencing PKC δ substantially prevented UV-stimulated cytochrome C concentration in cytosol, limited by our transfection efficiency ($\sim$ 65%; Figure 7E, left). The siRNA for PKC β had no effect in this regard. In contrast, silencing PKC ϵ partially reversed the inhibition of cytochrome C localization by E2 (Figure 7E, right). Again, the siRNA for PKC β had no effect, and PKC ϵ knockdown showed no influence on UV-induced cytochrome C release (in the absence of E2). Collectively, PKC δ contributes to UV-induced cell death via the mitochondria, whereas PKC ϵ specifically enables a cell survival-related action of the sex steroid.

Bax Translocation and Oligomerization at the Membrane

In response to many death stimuli, the proapoptotic protein Bax translocates to the mitochondria, where it undergoes oligomerization/activation and insertion into the outer mitochondrial membrane (Gross *et al.*, 1998; Dejean *et al.*, 2005). Active Bax then promotes the release of cytochrome C from the mitochondria into the cytoplasm, stimulating the formation of the apoptosome (Jurgensmeier *et al.*, 1998). We determined whether UV and E2 oppose each other in these regards. Under control conditions, Bax exists as a monomer in the cytosol of the MCF-7 cells (Figure 8A, lanes 1 and 5). UV caused both the dimerization and translocation of Bax to the mitochondrial fraction (lanes 2 and 6), significantly prevented by E2. Antioxidants prevented UV-induced translocation of Bax to the mitochondria (Figure 8B) and the PKC δ inhibitor rottlerin, substantially inhibited UV-induced Bax translocation (Figure 8C). JNK activation by UV also contributed, because the specific inhibitor of JNK1 activity, SP 600125, partially blocked Bax translocation to the mitochondria. Thus, the signaling that results from mitochondrial ROS formation leads to Bax participation in mitochondrial apoptosis, the inhibition of which provides a mechanism for E2 survival effects (Figure 8D).

Figure 5 (cont). study repeated a second time. (C) ER mediates E2 stimulation of MnSOD activity. MCF-7 cells or isolated mitochondria were exposed to brief UV and then incubated with 10 nM E2 in the presence or absence of 1 μ M ICI182780, for 20 min (left) or 4 h (right). The bar graphs represent three experiments combined. * p < 0.05 for control versus E2, + p < 0.05 for E2 versus same + either UV or ICI182780. Also shown is an immunoblot of MnSOD protein over 4 h in isolated mitochondria. (D) Left, validation of siRNA knockdown of MnSOD protein. Western blot for MnSOD or actin occurred 48 h after transfection of MCF-7 cells with either siRNA to MnSOD or GFP (control). Right, MCF-7 were transfected and recovered and then exposed to UV $\pm$ 10 nM E2. ROS were measured 4 h later and the data are from three combined experiments. * p < 0.05 for control versus UV, + p < 0.05 for UV versus UV + E2, ++ p < 0.05 for UV + E2 versus same plus siRNA to MnSOD.

exposed to UV $\pm$ E2 in the presence/absence of siRNAs, and cytochrome C was determined. Actin protein is expressed both as a specificity control for the effects of PKC knockdown and as a protein loading control. The studies were repeated a second time.

DISCUSSION

ER exist in the cytoplasm of many cells. However, it is unclear whether cytoplasmic receptors simply serve as a reservoir, awaiting signals for translocation to the nucleus or whether they function as a distinct pool. Previous studies suggested that estrogen impacts mitochondrial processes, perhaps underlying observed differences in the function of mitochondria from males and females (Borras *et al.*, 2003). Estrogen effects in the cell, however, might result from nuclear (or plasma membrane) actions of E2/ER indirectly impacting mitochondrial function (Wang *et al.*, 2003). Further, reports of mitochondrial actions of estrogen often utilized micromolar steroid concentrations, and so the physiological significance was unclear (Kipp and Ramirez, 2001; Morkuniene *et al.*, 2002).

Here, we provide evidence that ER α and ER β are present in the mitochondria of two different target cells for E2 action. In EC, both ER α and ER β exist predominantly in the nucleus, with some enrichment of ER α (compared with ER β) in mitochondria. In contrast, ER α is localized mainly to the nucleus of MCF-7 cells, and ER β is highly enriched in the mitochondria. These studies extend the findings of Chen *et al.* (2004), who reported that both ER isoforms are in the mitochondrial matrix of MCF-7, by immunogold labeling and electron microscopy. Other reports suggest that ER are present in the mitochondria of cardiomyocytes (Morkuniene *et al.*, 2002; Yang *et al.*, 2004), but this has been challenged in these cells (Forster *et al.*, 2004). Recently, ER α was identified in mitochondrial fractions from whole cerebral blood vessels (Stirone *et al.*, 2005). Importantly, we find that mitochondrial ER (and all cellular ER) are absent in EC isolated from combined ER α /ER β knockout mice. This novel finding indicates that mitochondrial ER are derived from the same genes that code for nuclear ER α and ER β .

Figure 7. PKC isoforms and cell fate. (A) Whole MCF-7 cells were exposed to brief UV $\pm$ E2, and then PKC δ activity was determined with phospho-specific antibody to tyrosine 311 (activation site) at 30 min and 4 h, during the presence or absence of sex steroid. PKC ϵ activity (serine 729 phosphorylation) was similarly determined. The study was repeated a second time, while specific PKC proteins were determined using separate antibodies to the PKC isoforms. (B) Cells were exposed to UV in the presence or absence of Mito Q and PKC δ activity was determined. A representative experiment of three and total PKC δ protein is shown. (C) PKC isoform activity. MCF-7 were incubated with 10 nM E2 in the absence or presence of UV exposure, the cells were lysed, and PKC δ or ϵ protein was immunoprecipitated and then utilized for an in vitro activity assay using appropriate substrate. Data are from a single study, repeated once. Total PKC protein is shown as a loading control. (D) MCF-7 were transfected with 2.5 μ g of siRNA to either PKC β , δ , or ϵ , and Western blotting for PKC δ (left) and PKC ϵ (right) occurred 48 h later. (E) Cells were transfected to express the various siRNAs, and experiments occurred 48 h later. Left, transfected and recovered cells were exposed to UV, and cytochrome C in cytoplasm was determined 4 h later. Right, cells were

Most importantly, we establish the first clear functions of mitochondrial ER potentially impacting breast cancer cell survival. Breast cancer cells expressing the mitochondrial-targeted E domain of ER α largely do not undergo several components of mitochondrial cell death (Kroemer, 2003). Interestingly, cells expressing plasma membrane-targeted E domain also show these cell survival actions of E2. As a G-protein-coupled receptor (Levin, 2005), the membrane-localized pool of ER might signal to the mitochondria. In this regard, PI3K activation by membrane ER contributes to breast cancer cell survival (Marquez and Pietras, 2001; Fernando and Wimalasena, 2004; Razandi *et al.*, 2004b), and PI3K and AKT suppress both Bax translocation to the mitochondria (Tsuruta *et al.*, 2002) and subsequent release of cytochrome C (Jurgensmeier *et al.*, 1998). We find that in cells expressing the E domain of ER α targeted to the cell membrane, wortmannin, a PI3 kinase inhibitor, partially reverses E2 prevention of cytochrome C release (unpublished observations). This suggests a cross-talk from membrane ER impacting mitochondria and cell viability.

In contrast, cells expressing a nuclear-targeted E domain or nuclear-targeted whole ER α do not support these cell survival actions. Nuclear ER importantly contribute to the long-term protection against cell death by transcribing anti-apoptotic genes such as Bcl-2 (Dong *et al.*, 1999). Shown here, the mitochondrial pool of ER blocked UV-induced cytochrome C release from mitochondria at 30 min postirradiation. Earlier mitochondrial events of apoptosis can occur as rapidly as 10 min after insult, with cells dying hours to days later (Green, 2005). Because the majority of our cells undergo apoptosis by 4–6 h post-UV exposure, the timing of our experiments is relevant.

We report that mitochondrial death/survival events in this setting are dependent on ROS formation. Specific compounds

Figure 8. Bax translocation to mitochondria (A) MCF-7 cells were exposed to UV $\pm$ E2. Bax localization and structure in cytosol and mitochondrial fractions were determined by Western blot using a specific antibody that detects BAX dimerization. The study was repeated two additional times. (B) MCF-7 cells were exposed to brief UV $\pm$ Mito-Q, NAC, or Rotenone for 4 h. Bax localization was then determined. The studies were repeated three times and the band intensity data were combined for the bar graph. * $p < 0.05$ for control versus UV, + $p < 0.05$ for UV versus same + antioxidant. (C) MCF-7 were exposed to UV $\pm$ 10 μ M SP 600125 (JNK 1 inhibitor), or 5 μ M Rottlerin (PKC δ inhibitor). The representative study was repeated. (D) Cartoon of estrogen action at mitochondria to prevent apoptosis.

that prevent ROS formation from mitochondrial respiratory complexes (Kelso *et al.*, 2001; Sato *et al.*, 2005) almost completely reverse UV-induced apoptosis. E2 up-regulated the activity of MnSOD, the SOD that only exists in mitochondria, and silencing MnSOD reversed E2-inhibition of ROS generation. Importantly, up-regulation of dismutase activity by E2 in isolated mitochondria and whole cells was comparable and rapid (10–20 min). This indicates that mediation of MnSOD activity

by E2 occurs mainly via mitochondrial ER. Precisely how this receptor pool signals to MnSOD activity requires further investigation of this novel finding. E2 also stimulates the transcription of the MnSOD gene in other cell systems (Strehlow *et al.*, 2003), prevented by ICI182780 and dependent on ERK MAP kinase (Borras *et al.*, 2005). We did not see significant changes in MnSOD protein in the mitochondria during the more limited, 4 h of E2 incubation with the cells.

What signals downstream of ROS mediate mitochondrial cell death? We found that ROS generated by UV induced PKC δ and JNK activities and E2 prevented this. PKC δ protein knockdown 1) partially prevented Bax translocation and oligomerization at the mitochondria and 2) prevented cytochrome C release and MCF-7 cell death. PKC δ overexpression contributes to apoptosis through association of the kinase with and modification of proapoptotic BCL-2 family members (Brodie and Blumberg, 2003; Murriel and Mochly-Rosen, 2003). In contrast, PKC ϵ has been implicated in the survival of cardiomyocytes (Gray *et al.*, 2004), prostate (McJilton *et al.*, 2003), and lung cancer (Ding *et al.*, 2002). Here we report the involvement of PKC ϵ in breast cancer cell survival induced by E2, limiting cytochrome C release. Further studies will be needed to delineate the precise functions of this kinase.

We also found that UV-induced ROS lead to JNK activation, JNK promoted Bax translocation and dimerization at the mitochondria, and E2 prevented each of these linked events. Radiation, chemotherapy, and tamoxifen activate JNK, contributing to breast cancer apoptosis (Mandlekar and Kong, 2001; Mingo-Sion *et al.*, 2004). ROS up-regulates JNK kinase activity by blocking JNK phosphatase function (Kamata *et al.*, 2005). Others (Tsuruta *et al.*, 2004) and we could not show that Bax is directly phosphorylated by this mitogen-activated protein kinase. Tsuruta *et al.* (2004) recently determined that JNK phosphorylates the 14-3-3 proteins that sequester BAX in the cytoplasm, releasing Bax to translocate to the mitochondria where it undergoes oligomerization and inserts into the mitochondrial membrane. This promotes pore formation and the release of cytochrome C into the cytoplasm (Jurgensmeier *et al.*, 1998). Inhibition of ROS-induced JNK provides a second mechanism by which E2 prevents Bax involvement in mitochondrial death.

Which mitochondrial ER isoform mediates E2 effects? Specific agonists for either ER α or ER β inhibited UV-induced cytochrome C release from isolated mitochondria; the ER β agonist was more potent. In addition, targeting of the ER α E domain only to the mitochondria supported several cell survival functions of E2. Thus, both receptor isoforms appear to contribute to sex steroid action in this organelle. Consistent with this, ER α or ER β agonists prevented UV-induced cell death, and steroid specificity was shown in that neither progesterone nor testosterone affected cell fate through mitochondria. Our results also suggest that the ligand binding/AF-2 region (E) is a functional domain for this pool of ER α .

Mitochondrial-generated ROS production may contribute to oncogenesis because cancer-causing mutations in mitochondrial DNA up-regulate ROS formation (Petros *et al.*, 2005). When produced in small amounts, ROS can serve as a proliferation-related signaling mechanism (Preston *et al.*, 2001). As recently reported, E2-induced G1/S progression in breast cancer may in part be related to this mechanism (Felty *et al.*, 2005), a mechanism well described for signaling by many growth factor tyrosine kinase receptors (Aslan and Ozben, 2003). In contrast, adjuvant therapies for breast cancer generate large amounts of ROS, essential to the induction of cell death (Benhar and Levitzki, 2002). Direct prevention by E2/ER of excessive mitochondrial ROS formation is a novel mechanism to prevent apoptotic cell death. Thus, it is the balance of ROS that mediates important aspects of carcinogenesis and provides a therapeutic target to alter tumor biology.

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