Tamoxifen-Induced Rapid Death of MCF-7 Breast Cancer Cells Is Mediated via Extracellularly Signal-Regulated Kinase Signaling and Can Be Abrogated by Estrogen

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Tamoxifen (Tam) is widely used in chemotherapy of breast cancer. It inhibits proliferation and induces apoptosis of breast cancer cells by estrogen receptor (ER)-dependent modulation of gene expression. In addition, recent reports have shown that Tam also has nongenomic effects. We previously reported induction of a rapid mitochondrial death program in breast cancer cells at pharmacological concentrations of Tam. Here we studied the upstream signaling events leading to mitochondrial disruption by Tam. We observed that 5 μM Tam rapidly induced sustained activation of ERK1/2 in ER-positive breast cancer cell lines (MCF-7 and T47D) and that PD98059 (inhibitor of ERK activation) was able to protect MCF-7 cells against Tam-induced death. These data suggest that activation of ERK has a primary role in the acute death response of the cells. In addition, inhibition of epidermal growth factor receptor (EGFR) opposed both Tam-induced ERK1/2 phosphorylation and cell death, which suggests that EGFR-associated mechanisms are involved in Tam-induced death. ERK1/2 phosphorylation was associated with a prolonged nuclear localization of ERK1/2 as determined by fluorescence microscopy with ERK2-green fluorescent protein construct. 17β-Estradiol was shown to exert a different kind of temporal pattern of ERK nuclear localization in comparison with Tam. Moreover, 17β-estradiol was found to oppose the rapid effects of Tam in MCF-7 and T47D cells but not in MDA-MB-231 cells, which implies a role for estrogen receptors in the protective effect of estrogen. The pure antiestrogen IC182780 could not, however, prevent Tam-induced ERK1/2 phosphorylation, suggesting that the Tam-induced rapid cell death is primarily ER-independent or mediated by IC182780 insensitive nongenomic mechanisms. (Endocrinology 148: 2764–2777, 2007)

TAMOXIFEN (Tam) IS a nonsteroidal selective estrogen receptor modulator widely used in the chemotherapy of breast cancer (1–3). It provides effective treatment for patients with metastatic breast cancer and reduces the risk of recurrence and death from breast cancer when given as an adjuvant therapy (4, 5). Use of Tam is especially indicated for postmenopausal women who have estrogen receptor (ER)-positive breast cancer. It is also being evaluated for use as a chemopreventive agent for women who have a high risk of developing breast cancer (6).

Tam acts primarily through ERs by modulation of gene expression. Whereas Tam at lower concentrations (0.1–1 μM) induces a cell-cycle arrest (7), pharmacological concentrations (above 5 μM) of Tam have been found to induce apoptosis (8) of breast cancer cells. Besides ER-mediated genomic effects, pharmacological concentrations of Tam have been shown to have ER-independent nongenomic effects in various cell types (9–11). Accordingly, Tam also has proapoptotic effects in ER-negative breast cancer cells and other cell types that lack ERs such as those in malignant gliomas, pancreatic carcinomas, ovarian cancers, and melanomas (12–15).

We have recently shown that Tam at pharmacological concentrations has an ionophoric effect on cell membranes associated with rapid changes in membrane permeability and intracellular pH, which leads to decreased viability and death of the cells (12). Other investigators have reported changes in membrane fluidity and alterations in intracellular calcium fluxes (11, 16) as well as increased oxidative stress and mitochondrial dysfunction in association with Tam-induced cell death (17–19). Tam has also been shown to rapidly inhibit estrogen-dependent protein kinase C in MCF-7 cells, HCC38 cells, and chondrocytes (20, 21) and activate the C-Jun N-terminal kinase pathway in IBE cells (22). Earlier work from our group also demonstrated that Tam induces a rapid mitochondrial death program in both ER-positive and ER-negative breast cancer cell lines (23).

Estrogens, in particular 17β-estradiol (E2), are well-characterized mitogens in mammary tissues and epithelial cells of the female reproductive tract. They also have an antiapoptotic influence in both ER-positive and -negative cells (24–27). This effect has also been previously reported in the ER-positive MCF-7 breast cancer cell line (28–30). However, the mechanisms of action are not fully defined. It has been shown that E2 induces transcription of the BCL-2 gene and increases translation of BCL-2 protein, which exerts antiapoptotic actions in many cell types (31, 32). In addition to nuclear events, estrogen has been demonstrated to be capa-
ble of bringing about rapid membrane-initiated signaling events in a variety of cell types (33). These include release of calcium, secretion of prolactin, generation of inositol triphosphate and nitric oxide, phosphorylation of Bad, and activation of MAPK and phosphatidylinositol 3-kinase/Akt (34–42). These findings support the hypothesis that estrogen can exert extranuclear actions either by interacting directly with growth factor receptors or through the recently described membrane-associated form of ER (43, 44). Cross talk from membrane-localized ERs to nuclear ERs has recently been proposed to be mediated through growth factor receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) (45). Growth factor receptors bring about signal transduction to kinases such as ERK that phosphorylate and activate nuclear ERs (46).

The aim of the present work was to characterize the upstream events leading to the previously observed mitochondrial disruption in breast cancer cells by Tam and study the effect of E2 on Tam-induced rapid death of breast cancer cells. In serum-free culture conditions, E2 was found to protect MB-231 cells were a gift from Dr. T. Guise (University of Texas Health Sciences, Little Rock, AR) were introduced into the MCF-7 cells using Endocrinology, June 2007, 148(6):2764–2777

2765

Zheng et al. • Role of ERK in Tamoxifen-Induced Rapid Cell Death

Science Center, San Antonio, TX). The cells were maintained in dimethylsulfoxide (DMSO) culture medium supplemented (10%) with iFBS and 2 mM t-glutamate. RPMI 1640, t-glutamine, and insulin were purchased from Sigma (St. Louis, MO), and FBS was purchased from Life Technologies, Inc. (Paisley, Scotland, UK). E2, Tam, and trypan blue solutions were obtained from Sigma, AG1478, and BIBX1382 were from Calbiochem (La Jolla, CA), PD98059 was from Molecular Probes (Eugene, OR), and ICI182780 was from Tocris (Ellisville, MO). All drugs were dissolved in DMSO (Sigma).

Cell death determination

Cells were cultured overnight in 3.5-cm-diameter tissue culture plates at a density of 1 × 10⁵ cells/plate, after which the culture media were replaced with phenol-red-free media containing dcFBS (5%) and 2 mM t-glutamate for 48 h. The cells were then treated with either 20 μM PD98059, 1 μM ICI182780, 10 μM AG1478, 10 mM BIBX1382, 10 mM E2, and/or Tam at the concentrations indicated in the results. Pretreatment times were either 1 h for PD98059 (20 μM), AG1478 (10 μM), and BIBX1382 (10 μM) or else 30 min or 4 h for ICI182780 (1 μM). Control plates had equivalent volumes of DMSO solvent. After treatment for 10–60 min, the cells were washed with PBS and cell death analysis was performed by way of trypan blue exclusion. Four to six separate areas of approximately 400 cells were assessed on each plate. The percentage of cells taking up blue dye determined relative cell viability.

Western blotting experiments

Cells were cultured overnight in 3.5-cm-diameter tissue culture plates at a density of 1 × 10⁵ cells/plate, after which the culture media were replaced with phenol-red-free media containing dcFBS (5%) and 2 mM t-glutamate for 48 h. The cells were treated for the various times indicated in the results and nontreated cells served as controls, these cultures having equivalent volumes of DMSO solvent. The cells were lysed in standard Laemmli sample buffer and lysates were sonicated for 10 sec and boiled for 5 min in a water bath at 100 C with β-mercaptoethanol. Aliquots (30 μl) of whole-cell lysate protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were blocked with 8% skim milk in Tris-buffered saline/0.05% Tween 20 and incubated with the primary antibodies for ERα, ERK1/2, and p-ERK1/2 (Cell Signaling, Beverly, MA) or β-actin (Sigma), and appropriate secondary antibodies. The proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) with colored markers (Bio-Rad, Hercules, CA) as size standards. Quantification of the bands was carried out with an MCID Image Analyzer (Image Research, Ontario, Canada). The relative pERK values were obtained from normalization of pERK1/2 values were normalized against the total ERK1/2 values.

Transient transfection and fluorescence and confocal microscopic imaging of the subcellular localization of ERK2

To investigate the subcellular localization of transiently expressed ERK2-green fluorescent protein (GFP) fusion protein, 5 × 10⁴ MCF-7 cells were grown in 3.5-cm-diameter petri dishes with coverslips in MCF-7 maintenance culture medium [(RPMI 1640 supplemented with iFBS (10), 2 mM t-glutamine, 10 mM E2, and insulin at 4 μg/ml)]. After 2 d, plasmids carrying GFP-ERK2 (a kind gift from Dr. Rony Seger, Department of Biological Regulation, The Weizmann Institute of Sciences, Rehovot, Israel) and red fluorescent protein targeted to the nucleus (nRFP, a kind gift from Dr. Stavros C. Manolagas, Division of Endocrinology and Metabolism, University of Arkansas for Medical Sciences, Little Rock, AR) were introduced into the MCF-7 cells using FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN), following the manufacturer’s instructions. The following day, the culture media were replaced with phenol-red-free media containing dcFBS (5%) and 2 mM t-glutamine. Transient expression of GFP-ERK2 protein was assessed 48–72 h after gene transfection by fluorescence microscopy. The cells were then treated with serum-free medium supplemented with drugs at the concentrations indicated in the results for 5, 10, 20, or 30 min. After treatment, the cells were fixed with 4% paraformaldehyde. Fluorescence imaging was carried out using an Ax-
iovert microscope with a ×63 oil-immersion objective (Zeiss, New York, NY) and an appropriate filter set. The percentage of cells showing nuclear accumulation of GFP-ERK2 was quantified by enumerating cells exhibiting increased GFP in the nucleus compared with the cytoplasm. Fluorescence of nRFP was used to visualize the nuclei. At least 20 fields (images of 1300 × 1000 pixels) selected by random sampling were examined for each experimental condition.

Statistical analyses

The statistical significance of differences between the control and treated cells were determined by the nonparametric Mann-Whitney and Kruskal-Wallis comparison tests. The differences between vehicle-pretreated cells and cells pretreated with PD98059, ICI182780, AG1478, or BIBX1382 were compared by ANOVA followed by multiple comparison tests. The critical value for significance was \( P < 0.05 \). Values are given as means ± se.

Results

\( E_2 \) opposes Tam-induced rapid death of MCF-7 and T47D cells but not MDA-MB-231 cells

To determine whether \( E_2 \) can oppose Tam-induced rapid death of breast cancer cells, ER-positive MCF-7 and T47D cells were first cultured in hormone-deprived medium for 48 h to eliminate the genomic survival effect of E2. The cells were then incubated in serum-free medium containing 1 \( \mu \)M, 2 \( \mu \)M, or 5 \( \mu \)M Tam for 10, 20, 30, 40, and 60 min, after which times cell death was determined by a trypan blue exclusion assay. Serum-free conditions were used because previous studies have shown that rapid effects can be most clearly demonstrated under such conditions (36, 38, 52–54). Control cells were treated with vehicle (0.2% DMSO) only. In the presence of 5 \( \mu \)M Tam the number of dead MCF-7 cells increased in a time-dependent manner (Fig. 1A). Nearly 50 and 90% of the cells were dead after 30 and 60 min treatment with Tam, whereas after treatment with both Tam and 10 nm \( E_2 \) for 30 and 60 min, the proportions of dead cells were only 10 and 20%, respectively. In the presence of 0.2% DMSO (vehicle), \( E_2 \) or Tam at concentrations of 1 or 2 \( \mu \)mol/liter, numbers of dead cells did not increase during 60 min of incubation.

To study the role of estrogen receptors in Tam-induced rapid death, the pure ER inhibitor ICI 182780 was added to MCF-7 cells at 1 \( \mu \)M concentration or a combination of 5 \( \mu \)M Tam and 1 \( \mu \)M ICI 182780 was used (Fig. 1B). As previously, approximately 40 and 80% of the MCF-7 cells were dead at 30 and 60 min after addition of 5 \( \mu \)M Tam alone. Addition of ICI 182780 with 5 \( \mu \)M Tam decreased cell death to some extent, the proportion of dead cells being approximately 25 and 60% after 30 and 60 min of incubation. ICI 182780 alone did not increase the number of dead cells during 60 min of treatment. The result suggests that ERs may, at least partly, be involved in the effect of Tam.

To test the effect of Tam on another ER-positive cell line we used T47D cells, which were first cultured similarly to MCF-7 cells. Tam (5 \( \mu \)M) caused death of approximately 50 and 80% of the cells at 30 and 60 min (Fig. 1C). Addition of \( E_2 \) with 5 \( \mu \)M Tam decreased the level of cell death, the proportion of dead cells being only 20% and nearly 40% after 30 and 60 min of incubation, respectively.

In the case of the ERα-negative and estrogen unresponsive MDA-MB-231 cell line (Fig. 1D), \( E_2 \) deprivation did not sen-

Tam induces rapid and sustained phosphorylation of ERK1/2 in estrogen receptor-positive breast cancer cells

To study the mechanism by which \( E_2 \) is able to protect MCF-7 cells from the effects of Tam, we first examined the activation of ERK1/2 by Western blot analysis. Unexpectedly, we found that Tam induced strong phosphorylation of ERK at 20 min of treatment (Fig. 2A). Tam (5 and 7 \( \mu \)M) yielded the effects that were approximately 4 and 5 times higher than ERK phosphorylation of nontreated control, respectively. At this time point, 10 nm \( E_2 \) did not induce phosphorylation of ERK. We then studied the effect of other treatment times on ERK phosphorylation. At the time point of 5 min, a transient increase of ERK phosphorylation was observed by mere addition of the vehicle, and no significant difference was observed between vehicle-treated cells and the cells treated with Tam and/or \( E_2 \). In our experimental setting, this is likely to be due to nonspecific signaling that results from replacement of the treatment medium at the 0 time point. Further studies demonstrated, however, that ERK phosphorylation in response to 5 \( \mu \)M Tam was increased (4-fold when compared with nontreated control) after 10 min, and this effect was sustained at least up to 40 min (Fig. 2B). At the time points of 20 and 40 min, Tam-induced ERK phosphorylation was approximately 4- and 3-fold, respectively, when compared with nontreated control. Phosphorylation of ERK in response to \( E_2 \) was observed at 10 min, although the change was not statistically significant at this time point. At later time points of 20 and 40 min, there was no ERK stimulation in \( E_2 \)-treated MCF-7 cells (Fig. 2B). However, \( E_2 \) was able to oppose Tam effect at 20 and 40 min when added together with Tam.

Prolonged ERK phosphorylation by Tam was also observed in ER-positive T47D cells (Fig. 2C). Addition of \( E_2 \) along with Tam did not, however, cause a statistically significant change in the level of Tam-induced ERK phosphorylation at 30 or 40 min. In ER-negative MDA-MB-231 cells, the difference between vehicle or Tam-treated cells at 5 or 30 min was not statistically significant (Fig. 2D).

Tam and \( E_2 \) induce different temporal patterns of ERK nuclear localization

Next we studied the ability of Tam to affect \( E_2 \)-induced nuclear localization of ERK. MCF-7 cells were transiently transfected with GFP-ERK2 and nucleus-targeted red fluorescent protein and exposed for different time periods to vehicle, \( E_2 \), Tam, and Tam plus \( E_2 \) before fixation and visualization by fluorescence microscopy (Fig. 3A). The localization of GFP-ERK2 was determined as the percentage of cells exhibiting accumulation of GFP-ERK2 in the nucleus (Fig. 3B). \( E_2 \) caused a rapid translocation of ERK to nuclei as previously reported in osteocytes (55). We found that in MCF-7 cells Tam not only induced a rapid and continuous
phosphorylation of ERKs, but it also brought about a rapid translocation of phosphorylated kinases to the nucleus. Whereas the E2-induced ERK nuclear translocation reached a peak level of approximately 60% at 5 min, returning to 30% at 10 min, Tam treatment resulted in increasing nuclear localization which reached a peak level of almost 60% at 20 min, returning to 30% at 40 min. Interestingly, Tam and E2 together resulted in a rapid and high level (over 70%) of nuclear translocation at 5 min, but most of the GFP-ERK2 had already returned to the cytoplasm at 10 min.

PD98059 opposes Tam-induced ERK phosphorylation and prevents Tam-induced rapid death of MCF-7 cells

To study Tam-induced ERK-phosphorylation further, we used PD98059, an inhibitor of MEK, at an ERK phosphorylation-inhibiting concentration (Fig. 4A). MCF-7 cells were incubated with either 20 μM PD98059 or DMSO vehicle for 1 h and then treated with 5 μM Tam for 20, 30, or 40 min, at which time points ERK phosphorylation was determined. PD98059 was found to oppose Tam-induced ERK phosphorylation completely, results that further confirm involvement of ERK in Tam-induced effects (Fig. 4B).

To determine whether Tam-induced phosphorylation of ERK is related to Tam-induced rapid death of breast cancer cells, MCF-7 cells were incubated with either 20 μM PD98059 or DMSO vehicle for 1 h and then treated with 5 μM Tam alone or Tam together with PD98059 for 10, 30, and 60 min. Dead cells were visualized by means of trypan blue exclusion assay. We found that PD98059 extended MCF-7 cell survival time in the presence of 5 μM Tam because a 30-min treatment with both 5 μM Tam and 20 μM PD98059 together resulted in a more than 75% decrease in the number of dead cells when compared with treatment with Tam only. After 60 min, the difference between the treatments was nearly 60% (Fig.
FIG. 2. Tamoxifen induces rapid and sustained phosphorylation of ERK in breast cancer cells. MCF-7 cells were grown for 2 d without E₂ and then incubated in serum-free medium containing DMSO (0.2%), 10 nM E₂, 1 µM Tam, 5 µM Tam, or 7 µM Tam for 20 min or left untreated (A). Alternatively, MCF-7 cells (B) and T47D cells (C) were similarly pretreated and incubated for 5, 10, 20, 30, or 40 min in the presence of DMSO (0.2%), 10 nM E₂, 5 µM Tam, or a combination of 5 µM Tam and 10 nM E₂ or left untreated. Whole-cell extracts were then prepared and aliquots of whole-cell lysate (30 µl each lane) were analyzed for ERK1/2 phosphorylation by Western blotting. In the case of MDA-MB-231 cells (D), whole-cell extracts were prepared from cells treated with DMSO (0.2%), 10 nM E₂, or 7 µM Tam for 5 or 30 min, respectively. The lower panels represent quantification of band intensities by means of an MCID Image Analyzer. Fold stimulations for A–C (compared with nontreated controls) and D (compared with vehicle control) were calculated. The columns represent values of three independent experiments (mean ± SE). For statistical analyses all treatments were compared with nontreated control (A–C) or vehicle control (D). *, P < 0.05 vs. control.
4C). The cultures treated with 20 μM PD98059 only or DMSO vehicle did not show an increased number of dead cells during 1 h of incubation.

**ICI182780 does not prevent Tam-induced ERK phosphorylation**

To study the role of ERs in Tam-induced rapid death, MCF-7 cells were cultured with either vehicle or ICI182780 for 30 min after which 5 μM Tam was added for 20, 30, and 40 min. Samples treated with either vehicle or 1 μM ICI 182780 for 40 min served as controls. As seen in Fig. 5, ICI182780 was not able to prevent Tam-induced ERK1/2 phosphorylation. At the time point of 40 min DMSO and ICI182780 (1 μM) did not induce phosphorylation of ERK.

**ERs partly oppose Tam-induced ERK phosphorylation and protect MCF-7 cells from Tam-induced rapid death**

To evaluate further the role of ERs in Tam-induced rapid death of breast cancer cells, we used a technique similar to that described by Marsaud et al. (56). They reported that a 1 μM concentration of the pure antiestrogen ICI182780 is able to degrade the ERs in MCF-7 cells. Accordingly, MCF-7 cells were cultured with ICI182780 or vehicle for 4 h before exposure to drugs. Figure 6A shows time-dependent degradation of ERα by ICI 182780. As demonstrated in Fig. 6B, in both vehicle- and ICI182780-treated cells 30 min of Tam-treatment induced a 6-fold and an almost 40-fold ERK phosphorylation, respectively, when compared with vehicle control. Treatment with a combination of E2 and Tam resulted in a decreased amount of cell death (approximately 75% decrease in vehicle pretreated cells and nearly 60% decrease in ICI 182780 pretreated cells) when compared with treatment with Tam only.

We also observed that ER-deficient (ICI182780 pretreated) MCF-7 cells were more susceptible to Tam-induced death (Fig. 6C) because the relative numbers of dead cells after 30 min of Tam treatment were approximately 50% among ER-deficient MCF-7 and nearly 60% among cells with an intact ER complement. A combination of Tam and E2 resulted in an approximately 50% decrease in ERK phosphorylation in comparison with treatment with Tam only. Cultures treated for 30 min with E2 only, ICI 182780, or vehicle after preincubation with either vehicle or ICI 182780 for 4 h did not show an increased number of dead cells.

**Inhibition of EGFR opposes both Tam-induced ERK1/2 phosphorylation and cell death**

The role of EGFR as a mediator of Tam-induced phosphorylation of ERK was studied by using AG1478, which is an inhibitor of EGFR and ErbB2. Cells pretreated with
AG1478 or vehicle were incubated with either 5 μM Tam alone or with a combination of Tam and E₂ for 30 min. Cells treated with DMSO or E₂ served as controls. As demonstrated in Fig. 7A, in vehicle-pretreated cells, 30 min of Tam treatment induced more than 40-fold ERK phosphorylation when compared with vehicle control. However, AG1478 almost completely opposed Tam-induced phosphorylation of ERK. To distinguish the effect of Tam between EGFR and ErbB2, we also used BIBX1382, which is an EGFR-specific inhibitor. As demonstrated in Fig. 7, A and B, inhibition of EGFR with BIBX1382 almost completely prevented Tam-induced phosphorylation of ERK in both MCF-7 and T47D cells, indicating the involvement of EGFR.

To determine whether inhibition of EGFR also has an effect on Tam-induced rapid death of breast cancer cells, MCF-7 cells were preincubated with either vehicle or AG1478, after which they were treated with serum-free medium containing 5 μM Tam or a combination of Tam and E₂ for 30 min. Control cells were treated with vehicle (0.2% DMSO) or E₂ only. Results of trypan blue exclusion analyses showed that inhibition of AG1478 extended MCF-7 cell survival time in the presence of 5 μM Tam because a 30-min treatment with both 5 μM Tam and 10 μM AG1478 resulted in a more than 50% decrease in the number of dead cells when compared with treatment with Tam only (Fig. 8A). As observed previously, E₂ extended the survival of cells because, in vehicle pretreated cells addition of E₂ yielded an approximately 70% lower relative number of dead cells than treatment with Tam alone. However, addition of E₂ along with 5 μM Tam and AG1478 did not further reduce the level of cell death.

We also evaluated the effect of BIBX1382 on Tam-induced rapid death of breast cancer cells. Trypan blue analyses demonstrated that, similarly to AG1478, the EGFR-specific inhibitor was able to significantly reduce Tam-induced death of both MCF-7 and T47D cells (Fig. 8, B and C). After a 30-min treatment of BIBX 1382-preincubated MCF-7 cells with 5 μM Tam, the relative number of dead cells was approximately
50% lower than in corresponding vehicle preincubated cells. Again, in vehicle-pretreated culture, combination of E2 and Tam resulted in a lower number of dead cells (H11011) when compared with treatment with Tam solely. However, addition of E2 along with Tam to BIBX 1382-preincubated cells did not further extend survival of the cells. In case of T47D cells, preincubation with BIBX 1382 reduced the level of cell death in Tam-treated cells approximately 30% when compared with vehicle preincubated cells. In vehicle-pretreated cells, combination E2 and Tam yielded a nearly 50% decrease in cell death when compared with treatment with Tam alone, but again in BIBX 1382-treated cells, E2 did not further decrease a number of dead cells.

**Discussion**

In this work we studied the receptors and signaling pathways involved in Tam-induced rapid death of breast cancer cells as well as the mechanisms by which E2 opposes the effects of Tam. These experiments were carried out under serum-free conditions that have commonly been used when studying rapid nongenomic effects (36, 38, 52–54). MCF-7
cells were also first cultured in hormone-deprived medium for 48 h to eliminate the genomic survival effect of E2. We observed that after estrogen deprivation Tam rapidly induced death of ER-positive MCF-7 cells, ER-positive T47D cells, and ER-negative MDA-MB-231 cells. E2 was able to oppose Tam-induced death of MCF-7 and T47D cells but not MDA-MB-231 cells, indicating the involvement of ERs in the survival action of E2 but not in rapid apoptotic effect of Tam. The protective effect of E2 against apoptosis-inducing agents in MCF-7 cells has been shown previously by Fernando and Wimalasena (41), who reported that E2 reduces apoptosis induced by TNF-α, H2O2, and serum withdrawal but not that induced by paclitaxel. However, the latter observation was in contrast to data published by Razandi et al. (52). A recent report of Pedram et al. (58) suggests that E2 inhibits UV radiation-induced apoptosis in MCF-7 cells by directly up-regulating manganese superoxide dismutase activity in these cells. In addition, the antiapoptotic action of E2 against resveratrol has been described by Zhang et al. (59). Nonetheless, even though E2 is able to oppose the effects of Tam, it is likely that E2 and Tam use separate signaling mechanisms.

It is speculated that Tam, besides its uptake to the cells and nuclear translocation, also generates a transmembrane signal transduction cascade by virtue of its high lipophilicity and partitioning in the cell membrane. Three major MAPK pathways exist in human tissues, but the one involving ERK-1 and -2 is most relevant to breast cancer (60). In our previous study (23), we demonstrated that Tam-induced rapid death of breast cancer cells was associated with an increase in production of reactive oxygen species. Because reactive oxygen species has been shown to contribute to cell death, in part, through an effect on various cellular signaling pathways including MAPK pathway (61–64), we tested the hypothesis that activation of ERK could be associated with Tam-induced rapid death of MCF-7 cells.

Activation of ERK has usually been considered to be in-
volved in cell proliferation (65). It has been suggested to be in inverse correlation to apoptosis (66, 67), and the role of ERK1/2 in cell death has only recently been hypothesized. Activation of ERK by Tam has previously been demonstrated in HeLa cells (68) and human endometrial cancer cells (69) but to our knowledge not in human breast cancer cells. Here we demonstrate that 5 µM Tam were able to activate ERK and that this activation was sustained for at least 40 min in MCF-7 cells, whereas in vehicle-treated cells, phosphorylation of ERK was detected only at 5 min of incubation. The transient increase of ERK phosphorylation was constantly observed at the time point of 5 min irrespective of the treatment with vehicle or E2 and/or Tam. It is possible that this nonspecific stress effect at 5 min covered the rapid ERK stimulation by E2 that has been demonstrated in several reports (41, 45, 70, 71) and possibly also that by Tam at 5 min. In this study, however, we focused our interest on the prolonged Tam effects at later time points at which no effects of vehicle control or E2 were observed.

Inhibition of ERK activation with PD98059 resulted in a decreased amount of cell death brought about by Tam, which results further demonstrate that Tam acts via ERKs to induce apoptotic signaling in MCF-7 breast cancer cells. The dual role of ERK1/2 in both cell death and cell proliferation may be explained by several recent findings that demonstrate that phosphorylated ERKs may produce different outcomes in the same cell, depending on the duration of ERK accumulation in the nucleus and perhaps also on cell context (55, 72–74).
Phosphorylation of ERK leads to translocation of activated ERK to nucleus. Nuclear translocation of MAPK is transient, although the duration time in the nucleus varies, depending on the cell types and the stimuli used (75). Our results from studies concerning subcellular localization of ERK after Tam treatment were basically in parallel with our phosphorylation data. Tam treatment induced a rapid nuclear localization of GFP-ERK, which increased up to the time point of 20 min. Consistently, phosphorylation of ERK was also elevated at 20 min. At 5 min, the combination of Tam and E2 led to an even higher nuclear accumulation of ERKs than did treatment with either Tam or E2 alone, which might be a result of cumulative activation of the ERK pathway by both compounds. Nuclear localization after Tam treatment began to decrease after 20 min, which was most likely the result of an increase of cell death after this time point. Accordingly, Tam-induced phosphorylation of ERK also decreased after the time point of 20 min. The proportion of nuclear GFP-ERK2 in control cells was already relatively high, approximately 30%, which might have been a consequence of overexpression of ERK in these cells. This may also explain the temporal differences between ERK phosphorylation and translocation.

Considering the observed kinetics of ERK nuclear translocation, the contribution of ERK activation to cell death by Tam may be further explained by the recent findings of Chen et al. on bone cells (62). They demonstrated that in osteoblasts, ERK phosphorylation may produce different outcomes in the same cell, such as proliferation vs. differentiation, depending on the duration of ERK phosphorylation and accumulation in the nucleus. They even show that the anti-apoptotic effect of E2 may be converted into a proapoptotic one by alteration of the temporal pattern the ERK activation, perhaps by determining the activation of a distinct set of transcription factors. Thus, it is possible that the localization and duration of kinase signaling similarly contribute to different actions of Tam in breast cancer cells. Persistent nuclear retention of activated ERK1/2 has also been considered as a critical factor in eliciting proapoptotic effects in neuronal cells subjected to oxidative stress (48). In our study, when compared with Tam, E2 treatment resulted in only transient activation of ERK and baseline conditions returned at 20 min. This is basically in parallel with the results of recent studies reporting that E2 rapidly and transiently activates ERK1 and ERK2 in MCF7 cells (41, 45, 70, 71). In these studies the peak of ERK activation is observed between 1 and 15 min. Thus, it is likely that in our experimental scheme, the transient activation of ERK by E2 is not well seen due to transient ERK activation caused by the replacement of treatment medium at the 0 time point. Moreover, there are also studies in which E2-mediated ERK activation in MCF-7 cells was not observed (76–78).

Rapid nongenomic ER-related signaling has been proposed to occur through distinct cellular localization of the classical ER or ER-like receptors, classical heterotrimeric G proteins, and many of the effectors traditionally associated with growth factors and G protein-coupled receptors (46). Some investigators postulate that nongenomic signaling of estrogen is mainly mediated by membrane-associated ERs (80), and this hypothesis has been further supported by the recent demonstration of membrane-located ERα in MCF-7 and other cell types (43, 81–83). Mechanisms of estrogen-mediated cellular actions have thus been shown to be very complex, and it is possible that Tam is also capable of activating various receptors and signaling pathways that are convergent.

In our study Tam was shown to activate ERK in ER-positive MCF-7 cells and T47D cells but not significantly in ERα-negative MDA-MB-231 cells, which results suggest the possible involvement of ERs (perhaps a membrane-associated ERα) in the induction of rapid death of breast cancer cells by Tam. However, our studies concerning the involvement of ERs in Tam-induced rapid cell death indicated that ICI182780 does not oppose Tam-induced ERK phosphorylation, which suggests independence of ERs. Independence of ERs was further supported by the finding that Tam also induces rapid death in ERα-negative MDA-MB-231 cells, even though not equally effectively. On the other hand, in a previous report, we have shown that the rapid effects of Tam are primarily ER independent but can be facilitated by ER-dependent mechanisms (23). Accordingly, we show here that ICI 182780 could partly oppose the effect of Tam in MCF-7 cells. Interestingly, a recent report by Heberden et al. (84) demonstrated a raft-located ER-like protein distinct from ERα, which remained insensitive to the pure estrogen antagonist ICI 182780. Another study recently showed the existence of a protein named ER-X, which could cross-react to an antibody directed against the binding site of ERα (85). Thus, it is possible that rapid effects of Tam in breast cancer cells are mediated by a yet unknown receptor structure. Although the reason for the lack of statistically significant ERK activation in MDA-MB-231 cells is not clear, activation of ERK seems to be important at least in the apoptotic response of ER-positive breast cancer cells against Tam. On the other hand, it is likely that Tam-induced rapid cell death is mediated only partly via the ERK pathway. However, it is notable that in MCF-7 cells addition of E2 along with Tam significantly opposed Tam-induced ERK phosphorylation, whereas this effect was not as clear in T47D cells. It is probable that the protective effect of E2 is mediated by ER. The discrepancy observed between MCF-7 cells and T47D cells may be due to differences in ERα to ERβ ratio between these two cell lines (86). The MCF-7 cells express a high ERα to ERβ ratio, whereas T47D cells express a low ERα to ERβ ratio, which might be relevant if the rapid effects of E2 are mainly mediated via the membrane-associated ERα as suggested by Pedram et al. (83).

In further studies involving the pure ER antagonist ICI182780, Tam-induced ERK phosphorylation was found to be stronger in MCF-7 cells in which ER was degraded as a result of ICI182780 treatment, suggesting that ER itself might somehow be able to protect breast cancer cells against Tam. However, it is more probable that pretreatment with ICI182780 sensitizes cells to the effect of Tam because ICI182780 alone was not able to induce rapid ERK phosphorylation or cell death. Surprisingly, E2 was found to oppose Tam-induced phosphorylation of ERK, even though ERs were degraded with ICI182780. In contrast to Marsaud et al. (56), Lippert et al. (87) recently suggested that a 4-h treatment with 1 μM ICI182780 does not completely degrade ER in MCF-7 cells. However, as demonstrated in Fig. 6A,
according to our results, ICI182780 treatment for 4 h was sufficient to completely degrade ERα. The reasons for this discrepancy could relate to subtle culture conditions and cell line variability. On the other hand, it is possible that Western blotting is not a method sensitive enough to detect low levels of ER that might still be left after an ICI182780 treatment. Use of serum-free conditions in our study might also enhance sensitivity of the cells to the effects of ICI182780. Another explanation for the effect of E2 is activation of rapid non-ER-mediated cellular signaling pathways by E2.

There is evidence that, in addition to acting via a membrane ER, estrogen can exert extranuclear actions by interacting directly with growth factor receptor complexes, which in turn leads to signal transduction to kinases such as ERK that phosphorylate and activate nuclear ERs (46). Shou et al. (88) demonstrated that when an EGFR family member ErbB2 was experimentally overexpressed, Tam treatment activated both ER and ErbB2 to signal downstream through ERK and phosphatidylinositol 3-kinase. However, this cross talk has been associated with resistance to endocrine therapy in breast cancer (57, 79), rather than induction of cell death. Nonetheless, our results suggest that Tam may also occupy growth factor signaling pathways for induction of cell death. We show that Tam-induced rapid death of breast cancer cells is, at least in part, mediated through EGFR because inhibition of EGFR totally abolished Tam-induced ERK-phosphorylation in both MCF-7 and T47D cells. Differences in growth factor receptor expression levels could also partly explain some discrepancies observed in responses of these two breast cancer cell lines to Tam treatment.

Cumulatively, the results of our work suggest that, in addition to previously demonstrated genomic effects, Tam is capable of acting via rapid membrane initiated signaling, which leads to death of breast cancer cells. Further work is needed to better understand possible relationships between nongenomic and genomic effects of Tam in breast cancer cells, but it is possible that these two mechanisms have different functions. Tam-initiated genomic signaling may represent a way through which the target cells are programed for complex functions that require a long time to get in action and ultimately determine the fate of the cells. Nongenomic signaling mechanisms, on the other hand, may represent systems by which cells are rapidly activated to adjust to dynamic changes of the cell environment. Identification of the multiple mechanisms underlying Tam-induced cell death is important because they could possibly be exploited to improve therapeutic responses to the treatment of patients with this selective estrogen receptor modulator.

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