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Delayed and Persistent ERK1/2 Activation Is Required for 4-Hydroxytamoxifen-induced Cell Death

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Abstract

Tamoxifen (Tam), and its active metabolite, 4-hydroxytamoxifen (OHT), compete with estrogens for binding to the estrogen receptor (ER). Tam and OHT can also induce ER-dependent apoptosis of cancer cells. 10–100 nM OHT induces ER-dependent apoptosis in ~3 days. Using HeLaER6 cells, we examined the role of OHT activation of signal transduction pathways in OHT-ER-mediated apoptosis. OHT-ER activated the p38, JNK and ERK1/2 pathways. Inhibition of p38 activation with SB203580, or RNAi-knockdown of p38 α , moderately reduced OHT-ER mediated cell death. A JNK inhibitor partly reduced cell death. Surprisingly, the MEK1/2 inhibitor, PD98059, completely blocked OHT-ER induced apoptosis. EGF, an ERK1/2 activator, enhanced OHT-induced apoptosis. OHT induced a delayed and persistent phosphorylation of ERK1/2 that persisted for >80 hours. Addition of PD98059 as late as 24 hours after OHT largely blocked OHT-ER mediated apoptosis. The antagonist, ICI 182,780, blocked both the long-term OHT-mediated phosphorylation of ERK1/2 and OHT-induced apoptosis. Our data suggests that the p38 and JNK pathways, which often play a central role in apoptosis, have only a limited role in OHT-ER-mediated cell death. Although rapid activation of the ERK1/2 pathway is often associated with cell growth, persistent activation of the ERK1/2 pathway is essential for OHT-ER induced cell death.

Keywords

Tamoxifen; Apoptosis; ERK; Estrogen Receptor

1. Introduction

When the clinically important Selective Estrogen Receptor Modulator (SERM), Tamoxifen (Tam), or its active metabolite, 4-hydroxytamoxifen (OHT), bind the estrogen receptor (ER), they induce a different ER conformation than the conformation induced by 17 β -estradiol (E₂) and other potent estrogens [1; 2]. In this conformation, the ER is less able to recruit

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[†]This paper is dedicated to our late friend and colleague, David Yu

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¹The abbreviations used are: Tam, Tamoxifen; OHT, 4-hydroxytamoxifen; ER, Estrogen Receptor; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; JNK, Jun N-terminal kinase; RNAi, RNA interference; SERM, Selective Estrogen Receptor Modulator; E₂, 17 β -estradiol; MAPK, Mitogen-activated protein kinase; PI, Propidium iodide.

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coactivators and may recruit corepressors [1; 3; 4]. By competing with E₂ and other estrogens for binding to the ligand binding pocket of ER, bound Tam and OHT may induce formation of an ER complex that is unable to effectively activate transcription of estrogen-regulated genes important in the growth and development of estrogen-dependent tumors. Microarray analysis in ER α positive MCF-7 human breast cancer cells and in MDA-MB-231 breast cancer cells stably transfected to express ER α indicate that Tam and OHT act as full or partial agonists on ~20% of the genes whose expression is directly regulated by E₂ [5]. Another group of genes is regulated by OHT-ER and not by E₂-ER [5], making Tam and OHT are partial, rather than pure, antagonists.

In addition to Tam's ability to arrest cell growth by preventing the growth promoting activities of E₂, Tam can induce death of both ER-positive and ER-negative cells [6; 7; 8; 9; 10]. Tam triggers apoptosis *in vitro* and shrinks some tumors *in vivo* [6; 7; 8; 9; 10]. Long term passaging of breast cancer cells results in resistance to tamoxifen [11]. In cells expressing very high levels of ER α , estrogens and other ER ligands induce cell death [12; 13]. Proposed mechanisms for tamoxifen-induced cell death include transcriptional regulation of Bcl-2 family proteins, activation of MAPK and other kinases through nongenomic pathways, and generation of an increase in intracellular Ca⁺⁺ concentration [7; 14; 15]. In many of these experiments, however, very high, μ M concentrations of Tam or OHT were used and some of the studies were carried out in cells that lack ER. To analyze these processes in cells that differ only in the presence or absence of ER, we analyzed Tam and OHT-induced cell death in ER-negative HeLa cells stably transfected to express hER α (HeLaER6: [16; 17]). We found that OHT induces cell death via two different pathways. When ER negative HeLa cells are maintained in medium containing 10–20 μ M Tam, OHT, E₂ or raloxifene, the cells die within 24 hours by a reactive oxygen-based pathway that triggers classical caspase-dependent apoptosis. Low, nM concentrations of OHT and sub-micromolar concentrations of Tam do not kill or damage ER-negative HeLa cells. When ER positive HeLaER6 cells are maintained in medium containing 1–100 nM OHT, they undergo apoptosis over several days. The antagonist: ICI 182,780, the SERM, raloxifene and E₂ protect the cells against OHT-ER mediated cell death [17]. The mechanism(s) by which OHT-ER induces cell death is largely unknown.

Liganded ER can exert its activities through genomic mechanisms and through non-genomic mechanisms based on rapid activation of signal transduction pathways [18; 19; 20]. Rapid activation of the ERK1/2 pathway by E₂-ER plays an important role in estrogen stimulation of cell growth [19; 21; 22; 23].

To analyze the role of signal transduction pathways in OHT-ER induced cell death, we examined the ability of OHT-ER to activate several signaling pathways. We then evaluated the effect of OHT-ER induced cell death by selectively inhibiting each signaling pathway. Although activation of the JNK and p38 pathways often plays a role in apoptosis, and OHT-ER activated both the JNK and p38 pathways, inhibiting activation of p38 and JNK had only a moderate ability to interfere with OHT-ER induced cell death. We found that OHT-ER induces a delayed and persistent activation of the ERK1/2 signaling pathway. Surprisingly, inhibiting activation of the ERK1/2 pathway blocked OHT-ER induced apoptosis. These studies describe a novel long-term OHT-mediated activation of the ERK1/2 pathway and demonstrate that this persistent activation of the ERK1/2 pathway is critical for OHT-ER induced apoptosis.

2. Experimental Procedures

2.1. Cell lines and cell culture

HeLaER6 cells (13, 14) were grown in Phenol Red-free DMEM (Sigma, St. Louis, MO) supplemented with 10% charcoal-dextran-treated fetal bovine serum and 150 µg/ml G418 (Invitrogen, Carlsbad, CA). Unless specifically mentioned, HeLaER6 cells were plated in Falcon 12-well plates (Becton Dickinson, Franklin Lakes, NJ) at 100,000 cells/well and grown for 24 h before treatment.

2.2. Assays for cell death and apoptosis

OHT was dissolved in ethanol and added to cultured cells at 1:1,000 v/v. The same volume of ethanol was added to control cells. Unless otherwise indicated, cells were harvested 72 h after addition of OHT. The adherent cells were harvested by incubating with PBS containing 1 mM EDTA for 5 min at room temperature. The harvested, formerly adherent, cells were combined with the dead cells and late-stage apoptotic cells that were floating in the culture medium. The loss of mitochondrial membrane potential was detected by staining with DiOC₆(3) (Molecular Probes, Eugene, OR) as previously described [17]. Dead cells were detected using propidium iodide ([PI], Molecular Probes). Briefly, cells were sedimented by centrifugation at 400 x g for 3 min., resuspended in 0.7 ml PBS containing 40 nM DiOC₆(3), incubated in a 37°C water bath for 15 min and then placed in an ice bath. PI was added to each sample to 1 µg/ml 2 min before the sample was assayed on a Coulter XL flow cytometer. Filters at 525 nm and 620 nm were applied for DiOC₆(3) and PI detection, respectively.

Fragmented chromosomal DNA was detected using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay kit (APO-BRDU Kit, Phoenix Flow Systems, San Diego, CA). Cells were grown and treated in 6-well plates, harvested, washed once with PBS, fixed with 1% paraformaldehyde on ice for 15 min, and stored in 70% ethanol at -20°C for >10 h. The labeling of Br-dUTP on the ends of DNA fragments and the reaction with the fluorescent dye labeled anti-BrdU antibody were performed as suggested by the supplier. Apoptotic cells (BrdU positive) were sorted using flow cytometry.

2.3. Blocking the activity of enzymes in signaling pathways with specific inhibitors

MEK1/2 inhibitor PD98059 (Cell Signaling, Beverly, MA), p38 inhibitor SB203580 (Tocris/Cookson, Ellisville, MO), JNK inhibitor SP600125 (Tocris) and general caspase inhibitor Z-VAD-FMK (BIOMOL Research Labs., Plymouth Meeting, PA) were from the indicated suppliers. The inhibitors were dissolved in DMSO and diluted to concentrations that could be added to the cultured cells at 1:1,000 v/v. PD98059 was added to the medium at 1:500 v/v. The cells were incubated with the inhibitors 1 h before, or at the indicated time after the addition of 100 nM OHT. Cell viability was assayed 72 h after addition of OHT.

2.4. Western blotting

The cells were homogenized by adding 50 µl–100 µl of RIPA buffer (20 mM Tris-HCl, pH7.4, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium orthovanadate, 100 µM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin) to each well of 12-well plates and incubating on ice for 15 min. Cell lysates were transferred to microcentrifuge tubes and cell debris was sedimented by centrifugation at >9,000 x g for 5 min. Protein concentrations were determined using Coomassie Blue reagent (Bio-Rad, Richmond, CA). The cell lysates were mixed with 3x loading buffer and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Specific protein bands on these transferred membranes were detected using the following antibodies: ERK1/2 and phospho-ERK1/2,

p38 (C-20, no cross-reactivity with p38 β and p38 γ) and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies for phospho-p38, JNK and phospho-JNK were from Cell Signaling (Beverly MA). Protein bands bound to antibodies were visualized by staining with ECL Plus (Amersham Bioscience, Piscataway, NJ) and data was recorded with a Molecular Dynamics Storm PhosphorImager (Amersham Bioscience, Piscataway, NJ).

2.5. RNA interference

The 21 nucleotide siRNA for human p38 α mRNA (NCBI Accession # L35253; starts at amino acid 1246) is 5'-AACCAGUGGCCGAUCCUUAUG(TT)-3' (Dharmacon, Lafayette, CO). 6 μ l of a 20 μ M stock solution of p38 α siRNA was transfected using Oligofectamine (Invitrogen) into ~60,000 cells/well grown in a 12-well plate. The expression of p38 α protein was detected by Western blotting of cell lysates prepared 48 and 72 h after transfection using an anti-p38 α antibody (Cell Signaling). The effect of knockdown of p38 α on OHT-ER-mediated cytotoxicity was examined by treating HeLaER6 cells with OHT 48 hours after the transfection. Cell viability was analyzed by flow cytometry.

3. Results

3.1. OHT-induced apoptosis is slow

We previously showed that <1 μ M OHT and Tam induce ER-dependent programmed cell death [17]. We determined the time course and concentration-dependence of OHT-ER-induced cell death, and we evaluated cell death using several widely used apoptosis assays. Propidium iodide (PI) passes through the damaged cytoplasmic membranes of dying and dead cells, binds to chromosomal DNA and fluoresces. Low concentrations of the strong cationic fluorescent dye, DiOC₆(3), accumulate in the intact mitochondrial membranes of cells that are not undergoing apoptosis. The reduced mitochondrial membrane potential that often occurs during apoptosis results in reduced binding of DiOC₆(3) and a decline in fluorescence intensity. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay detects cells with the fragmented chromosomal DNA that is a hallmark of caspase-dependent apoptosis.

HeLaER6 cells were maintained in medium containing 0.1 nM -10 μ M OHT for 24, 48, 72 and 96 hours. The cells were harvested then stained with PI (Fig. 1A), DiOC₆(3) (Fig. 1B), or labeled using TUNEL assay kits and then analyzed by flow cytometry (Fig. 1C). Although the cells maintained in medium containing sub-micromolar concentrations of OHT exhibited significant morphology changes at earlier times (data not shown), apoptosis was not detectable until two days post-treatment. After 48 hours in medium containing >1 nM OHT, 20–30% of the cells were dead, as measured by the PI positive and low DiOC₆(3) staining cell populations (Figs. 1A and 1B). Consistent with our previous data indicating that cells killed by OHT within 48 hours do not exhibit all of the hallmarks of caspase-dependent apoptosis [17], no TUNEL positive population was detected at 24 or 48 hours in cells maintained in 10 nM OHT (Fig. 1C, 10 nM OHT). As positive controls, we used 20 μ M OHT, which activates ER-independent apoptosis [17], and 12.5 μ M etoposide, a topoisomerase II inhibitor widely used to induce apoptosis [17]. 20 μ M OHT and 12.5 μ M etoposide turned more than 50% of the cells TUNEL positive within 24 h of treatment (Fig. 1C, open bars). After 96 hours in medium which contained nanomolar concentrations of OHT, 60–90% of the OHT-treated cells were dead as measured by PI staining, DiOC₆(3) and TUNEL (Fig. 1, panels A–C).

Caspases play key roles as both initiators and effectors in most apoptotic pathways [24]. Z-VAD-FMK is an irreversible inhibitor of a broad range of caspases, including caspases 1, 3, 4 and 7. To determine whether caspases are involved in OHT-induced cell death, we added 25 μ M and 50 μ M Z-VAD to HeLaER6 cells 1 h before and 4, 24 and 48 h after treatment

with 100 nM OHT. Both 25 μ M and 50 μ M Z-VAD-FMK largely blocked OHT-ER induced apoptosis, even when the Z-VAD-FMK was added 48 hours after OHT (Fig. 1D).

3.2. Caspases 3 and 9 are activated in OHT-ER-induced apoptosis

To more directly evaluate the roles of caspases 9 and 3 in OHT-ER mediated apoptosis, we looked at their ability to cleave caspase-specific peptides that fluoresce only after cleavage. After 72 hours there were a few fluorescent cells in the control population. The cells maintained in medium containing OHT exhibited robust activation of caspases 9 and 3 (Fig. 2). These results indicate that OHT-ER kills cells by inducing caspase-dependent apoptosis.

3.3. OHT-ER activation of p38 plays a moderate role in OHT-ER-induced apoptosis

p38 and JNK are members of the MAP kinase family and are involved in multiple intracellular death pathways [25; 26; 27; 28; 29]. P38 and JNK are activated by environmental stresses and by some anti-tumor agents [30]. Using a specific pharmacological inhibitor of p38, SB203580, we previously suggested a role for p38 in OHT-induced cell death [12]. Significantly increased phosphorylation of p38 (P-p38) was detected in HeLaER6 cells treated with 100 nM OHT at all times tested (4–72 hours) (Fig. 3A).

To further examine the role of p38 in OHT-ER-induced cell death, we knocked down p38 α using a p38 α -specific siRNA and then treated the cells with OHT. Western blot analysis demonstrated that transfecting HeLaER6 cells with the p38 α -specific siRNA significantly reduced the level of p38 α protein (Fig. 3, panels B and C). Compared to cells transfected with the control pGL3 luciferase siRNA, in multiple independent experiments, knockdown of p38 α resulted in a $38 \pm 7\%$ ($n=4$, $P=0.007$ by Students T test) reduction in the number of cells undergoing OHT-ER mediated apoptosis (Fig. 3D, p38/OHT).

3.4. Inhibition of p38 with SB203580

To confirm that the p38 inhibitor, SB203580, was functioning in our system, we tested its ability to block OHT-induced phosphorylation of p38. SB203580 effectively blocked the OHT-induced phosphorylation of p38 (Fig. 4A). Combining p38 α knockdown with treatment with SB203580 did not significantly increase the percentage of cells protected against OHT-ER-induced apoptosis compared to either treatment alone (Fig. 4B, p38 and p38/SB). These data indicate that activation of the p38 pathway is not likely to be the key factor that mediates OHT-ER-induced cell death. Since activation of the p38 pathway played only a moderate role in OHT-ER induced cell death, we examined the role of activation of the JNK pathway.

3.5 Activation of the JNK pathway plays a moderate role in OHT-ER-induced apoptosis

To analyze JNK activation, we carried out Western blotting with a phospho-JNK-specific antibody. There was a moderate increase in the level of P-JNK protein from 4 to 72 hours after addition of OHT to the medium (Fig. 5A). We also tested the ability of the JNK inhibitor, SP600125 (SP), to interfere with OHT-ER-induced apoptosis. SP is a reversible inhibitor that is competitive with ATP and exhibits a >10X higher affinity for JNK-1, -2 and -3 than for other serine/threonine kinases [31]. At 25 μ M SP has a moderate effect in blocking induction of apoptosis by OHT (Fig. 5B). Western blotting demonstrated that pretreatment with 25 μ M SP clearly prevented JNK phosphorylation (Fig. 5C). These data are consistent with the view that activation of the p38 and JNK pathways plays a modulatory role and is probably not central to OHT-ER-induced apoptosis.

3.6. Inhibiting ERK1/2 activity blocks OHT-ER-induced apoptosis

ERK1/2 are two structurally and functionally related proteins that play a critical role in intracellular signaling by a large and varied group of growth factors and cytokines [30]. Although activation of ERK1/2 influences diverse pathways and cell processes, many of the best-characterized outcomes of ERK1/2 activation involve stimulation of cell proliferation [32; 33; 34; 35; 36]. Rapid and short-term activation of ERK1/2 is an extensively studied and well characterized non-genomic activity of E₂ and SERMs [21; 37; 38; 39; 40; 41; 42]. We therefore investigated the role of ERK1/2 in OHT-induced cell death.

PD98059 (PD) is a highly specific and widely used inhibitor of MEK1/2 that effectively blocks the activation of ERK1/2, the downstream substrate of MEK1/2 [43]. To investigate the role of the ERK1/2 pathway in OHT-ER-induced apoptosis, we pre-incubated the cells with 2 μ M, 10 μ M and 50 μ M PD for 1 h, and then maintained the cells in medium containing 100 nM OHT for 72 h. Since ERK1/2 activation is often associated with cell proliferation and is usually anti-apoptotic [44; 45], we expected enhanced OHT-induced apoptosis in the cells pretreated with PD98059. Surprisingly, the MEK inhibitor elicited a dose-dependent protection against OHT-ER induced cell death. At the highest concentration tested, 50 μ M, PD98059 nearly abolished OHT-ER induced apoptosis (Fig. 6A).

To determine whether the effect of PD98059 was specific for OHT-ER-induced apoptosis, we compared the effect of PD98059 in cells incubated in 100 nM OHT and 1 μ M TAM, which induce apoptosis only through the ER-dependent pathway, 20 μ M OHT, which activates ER-independent apoptosis through a classical reactive oxygen pathway, and with etoposide [17]. PD98059 blocked apoptosis induced by 100 nM OHT or by 1 μ M Tam (Fig. 6B). The antagonist ICI 182,780 completely blocked apoptosis induced by low concentrations of OHT and Tam but had no effect on ER-independent apoptosis induced by 20 μ M OHT or by etoposide [17]. Therefore, PD98059 and ICI 182,780 both completely blocked apoptosis induced by low concentrations of OHT and Tam. Neither PD98059 nor ICI 182,780 had any effect on apoptosis induced by etoposide or by 20 μ M OHT (Fig. 6B). These results strongly suggest that an activated ERK1/2 pathway is required for ER-dependent, OHT-induced apoptosis in this system. Inhibiting ERK activation has no effect on classical apoptosis induced by etoposide and by μ M concentrations of OHT.

3.7. OHT induces a delayed and persistent activation of ERK1/2 that is essential for OHT-ER-induced apoptosis

ERK1/2 are activated by MEK1/2 through phosphorylation of Thr202/Thr204 [46]. We assayed ERK1/2 activation by OHT and the inhibition of ERK1/2 activation by PD using Western blotting with phospho-Thr202/Thr204-specific antibody. Most studies of the effects of estrogens and Tam on the ERK1/2 pathway have focused on rapid and transient activation of the ERK1/2 pathway. However, our observations that OHT-ER-induced apoptosis requires several days and is prevented by addition of Z-VAD FMK 48 hours after exposure of the cells to OHT (Fig. 1D) led us to examine ERK1/2 phosphorylation over a broad range of times.

From 1–24 hours after addition of OHT to the medium, we did not observe a consistent increase in ERK1/2 phosphorylation. Increased ERK1/2 phosphorylation was consistently detected 48 hours after addition of OHT and continued for up to 84 h (Fig. 7A, 48 and 72 h, OHT, -PD). Of course, at 72 and 84 hours post-OHT addition, there was also extensive OHT-ER-induced apoptosis of the cells. Pre-incubation of the cells with 50 μ M PD dramatically reduced ERK1/2 phosphorylation. Interestingly, 10⁻⁸ M E₂, which does not kill the cells, also induced a PD98059 sensitive, long-term, activation of ERK1/2. E₂ activation of ERK1/2, but not OHT activation, could be detected at 12 hours (Fig. 7A). The levels of

total ERK1/2 did not change in a systematic way over time, and an increase in the level of ERK1/2 was not responsible for the increased level of phospho-ERK1/2 seen at 48 and 72 hours (Fig. 7A).

Since OHT induces a delayed, long-term activation of ERK1/2, and ZVAD-FMK could protect the cells from OHT-induced apoptosis even when added 48 hours after OHT, we examined the ability of PD98059 to protect the cells after prior exposure of the cells to 100 nM OHT. We treated the cells with 50 μ M PD98059 1 hour before addition of OHT (–1 hour) and 1, 4, 12, 24 and 48 hours after addition of OHT. PD98059 affords about the same level of protection from OHT-induced apoptosis when added from 1 hour before OHT to 12 hours after OHT. OHT-ER-induced apoptosis was still reduced by 50% when PD was added 24 h after OHT. At 48 hours after addition of OHT, the cells had largely become committed to apoptosis and PD98059 only reduced the extent of OHT-ER-induced apoptosis by ~20% (Fig. 7B). These data indicate that OHT elicits a delayed and persistent activation of ERK1/2, which is necessary for OHT-ER induced apoptosis.

If ERK1/2 activation is indeed required for OHT-ER-induced apoptosis, then activators of ERK1/2 might enhance OHT-ER mediated apoptosis. Since the delayed and long-term activation of ERK1/2 elicited by OHT is quite novel, there were no readily available ERK1/2 activators that could mimic the effect of OHT. We therefore analyzed the effect of the powerful transient ERK1/2 activator, epidermal growth factor (EGF) [47; 48].

3.8. The ERK activator, epidermal growth factor- α (EGF- α), enhances OHT-ER-induced apoptosis

To test the idea that activation of ERK1/2 by EGF- α could enhance OHT-ER-induced cell death, we added 100 ng/ml EGF- α to the medium 1 h before or 24 hours after adding OHT. The percentage of the cells undergoing apoptosis was measured 60 hours later. EGF- α alone did not change the small percentage of the control cells undergoing apoptosis (Fig. 8, none and EGF, –1h and 24h). Compared to cells exposed to OHT alone, addition of EGF 1 hour before OHT increased the percentage of cells undergoing OHT-ER-induced apoptosis by 53% (Fig. 8, OHT and OHT/EGF –1h). Addition of EGF 24 hours after OHT had a much smaller effect on OHT-mediated apoptosis (Fig. 8, OHT/EGF 24h).

Previous studies indicated that activation of ERK1/2 by EGF could enhance ER-mediated transactivation [49; 50; 51; 52; 53]. Activation of ERK1/2 appears to induce a functionally important phosphorylation of ER α at Ser 118 [54] and may result in phosphorylation and activation of ER coactivators [55; 56; 57; 58]. At 4 hours after exposure of the cells to EGF + OHT, there was a ~2 fold increase in phosphorylation of Ser118 of ER α . Consistent with the data showing that OHT induces a delayed activation of ERK1/2 (Fig. 7A), OHT alone did not induce phosphorylation of ERK1/2 or Ser118 of ER α at 4 hours (data not shown). EGF induced the expected strong increase in ERK1/2 phosphorylation at 15 min. An EGF-mediated increase in ERK1/2 phosphorylation was still detectable 4 hours after EGF addition. These data are consistent with the idea that EGF- α enhances OHT-ER-induced cell death by activating the ERK pathway.

3.9. ICI 182,780 blocks the OHT-ER-mediated long-term activation of ERK1/2

Our finding that persistent activation of the ERK1/2 pathway was essential for OHT-induced cell death led us to examine the question of whether ER α was responsible for the long-term activation of the ERK1/2 pathway. Although the issue remains controversial, there have been several reports that E₂, OHT and ICI 182,780 can all bind to the G-protein GPR30 and thereby rapidly activate the ERK1/2 pathway [59; 60; 61; 62]. Since these studies indicated that ICI 182,780 bound to GPR30 could rapidly activate the ERK1/2 pathway, and our work

([17], Fig. 5B) showed that excess ICI 182,780 efficiently blocked OHT-induced apoptosis, we examined the effect of ICI 182,780 on the long-term activation of ERK1/2 induced by OHT and by E₂. Excess ICI 182,780 blocked the long-term activation of ERK1/2 by OHT and by E₂. (Fig. 9, P-ERK, compare 24h E₂ and OHT –ICI and +ICI). These data are consistent with the view that it is the binding of OHT to ER α that is responsible for both the long-term OHT-mediated activation of ERK1/2 and for OHT-induced apoptosis. Alternatively, the different effects of ICI 182,780 may reflect the presence of different cell backgrounds.

4. Discussion

Sub-micromolar concentrations of OHT activate the p38, JNK and ERK1/2 pathways. Although activation of the p38 and/or JNK pathways plays a key role in apoptosis induced by UV light, osmotic stress and several other inducers of apoptosis [30], inhibition of the p38 and JNK pathways had only a moderate effect on OHT-induced cell death suggesting that these pathways make modest contributions to OHT-induced cell death. Inhibition of ERK1/2 activity with PD98059 blocked apoptosis induced by 10–100 nM OHT and by 1 μ M Tam. Activation of ERK1/2 by EGF enhanced OHT-mediated apoptosis. Based on these data, we focused primarily on the ERK1/2 pathway. Other pathways that were not evaluated, including the protein kinase A pathway, could also influence OHT-induced apoptosis, either directly or by modulating activation of the ERK1/2 pathway.

Although ERK1/2 activation is commonly associated with cell proliferation and is usually viewed as anti-apoptotic, prolonged activation of ERK1/2 is responsible for cell death in a few systems. In the mouse hippocampal cell line, HT22, glutamine excitotoxicity required prolonged exposure to activated nuclear ERK1/2 [63]. Transfecting an OHT-activated er-b-raf fusion into hepacytes resulted in phosphorylation and activation of ERK1/2 for more than 36 hours and led to inhibition of DNA synthesis [64]. Duration of ERK phosphorylation plays an important role in the establishment of diverse cell signaling decisions following ERK1/2 activation (reviewed in: [46; 65]).

Binding of high affinity ligands to ER induces a rapid and transient nongenomic phosphorylation of ERK1/2 [66; 67; 68; 69; 70]. Several sites at which ER acts on the ERK1/2 pathway have been identified, suggesting that there may be different modes of ER activation of ERK1/2 in different cell and regulatory contexts [70; 71; 72]. ER-agonist binding in the cytoplasm enables direct contact with Src, which in turn recruits Shc and triggers the Ras/MEK/ERK signal cascade. The formation of this complex is likely facilitated by scaffolding proteins, including MNAR [70; 73] and caveolin [74; 75]. The E₂-ER α complex, or E₂ alone, may also cause the release of heparin-bound EGF through the mediation of a G protein-coupled receptor, GPR30, and Src. The released EGF could then activate EGF receptors, which are potent activators of the ERK1/2 pathway [62; 76]. IGF-1R is a growth factor receptor that interacts with ERK1/2. IGF-1R reportedly binds membrane-associated ER, which stimulates IGF-1R activation of ERK1/2 [77]. In addition, ERK1/2 can be activated by increased intracellular Ca⁺⁺ that is triggered by the binding of E₂ or TAM to G protein receptors [7; 78].

After addition of OHT to the medium, we detect a delayed ERK1/2 phosphorylation after 12–48 h, which persists for up to 86 h. The MEK inhibitor, PD98059, blocks OHT-induced apoptosis, even when it is added 24 h after OHT treatment. These data suggest involvement of a genomic pathway in ER-dependent, OHT-induced cell death.

ICI 182,780 blocks OHT-ER-induced apoptosis and the persistent OHT-ER induced phosphorylation of ERK1/2. However, E₂ induces persistent activation of ERK1/2 but does not induce apoptosis. Since levels of Bcl-2 and Bcl-XL were similar in HeLaER6 cells

treated with E₂, or with OHT (data not shown), induction of these antiapoptotic proteins [79; 80] is not responsible for the failure of E₂ to induce apoptosis. Since pre-treatment with the Akt inhibitor, wortmannin (a specific inhibitor of PI-3 kinase), did not result in E₂ becoming pro-apoptotic (data not shown), E₂ activation of PI-3 kinase may not be responsible. Although E₂ and OHT both induce long-term activation of ERK 1/2, E₂ activation generally appears earlier than OHT (Fig. 7A) and this may play a role in the different effects of E₂ and OHT on apoptosis. The most straightforward explanation is that long-term activation of ERK1/2 by E₂ and by OHT facilitates the ability of E₂-ER and OHT-ER to activate or repress the transcription of different genes with very different effects on apoptosis. Microarray studies show that OHT-ER activates and represses expression of a substantial number of genes that show little or no response to E₂-ER [5].

The OHT-ER induced changes that ultimately lead to apoptosis occur slowly over 2–3 days. Although the loss of membrane potential and the leakage of cytochrome C suggest mitochondrial damage at 48 hours, markers of the later stages of apoptosis, such as caspase activation and DNA fragmentation, are generally not observed until after 3 days of OHT treatment.

Our data is consistent with a three-step model for OHT-ER induced apoptosis. In the initial step, OHT-ER acts at the transcriptional level and regulates the production of mRNAs encoding protein(s) that are important in prolonged ERK1/2 activation. This persistent activation of ERK1/2 has several consequences. Long-term ERK1/2 activation may modify cell functions, such as drug resistance, and alter cell architecture and cell morphology [81; 82]. ERK1/2 activation also results in phosphorylation of ER and ER coactivators [54; 55; 56; 83], thereby increasing the transcriptional potential of OHT-ER. The phosphorylated ER may induce expression of mRNAs coding for pro-apoptotic proteins, or directly or indirectly repress expression of anti-apoptotic proteins. Long-term activation of ERK1/2 may lead to an increase in intracellular stress that helps trigger apoptosis, as suggested in H₂O₂-induced apoptosis of human glioma cells [84]. Inhibition of stress-related kinases, such as p38 and JNK, can only attenuate the cell stress and does not completely block the process leading to apoptosis.

Our data shows that delayed and persistent ERK1/2 activation is necessary for ER-dependent, OHT-induced cell death. This strongly suggests that Tam-ER and OHT-ER can elicit long-term effects that can lead to apoptosis in addition to their widely reported rapid non-genomic effects. The pro-apoptotic pathway activated by OHT-ER likely involves a combination of effects mediated at the transcriptional level and effects due to the long-term activation of ERK1/2, which in turn modifies the transcriptional potential of TAM-ER and OHT-ER. The discovery of delayed and persistent activation of ERK1/2 as a critical activity of OHT-ER indicates a new type of reciprocal cross-talk between liganded ER and signaling pathways.

Long term ERK activation may have a role in other events that involve ER-mediated transcription. Transient transfections in the presence of pathway-specific inhibitors or ER and coactivator mutants defective in specific phosphorylation sites demonstrate alterations in ER-mediated transactivation several days after initial hormone administration [21; 54; 85]. The long-term activation of ERK1/2 by E₂-ER and by OHT-ER may help to explain these diverse observations. Our observation of a functionally important, delayed and persistent activation of ERK1/2 by E₂ and by the SERM, OHT, enhances and extends the many important studies demonstrating rapid activation of ERK1/2 by E₂-ER.

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FIG. 1.

OHT kills cells by slow induction of caspase-dependent apoptosis. In panels A and B HeLaER6 cells were maintained in medium containing either the ethanol vehicle or the indicated concentrations of OHT and harvested after 24, 48, 72 and 96 h. Cell death was analyzed by flow cytometry using propidium iodide (panel A), DiOC₆(3) (panel B), or terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) (panel C) as described in Experimental Procedures. Assay reproducibility can be judged from the plateau data from 10 nM to 20 μ M. The data in panels A–C is representative of apoptosis levels seen in additional experiments. In panel D, 25 μ M or 50 μ M of the general caspase inhibitor Z-VAD-FMK was added to cell cultures 1 h before or 4, 24 and 48 h after OHT treatment. Flow cytometry was used to detect apoptotic cells as low DiOC₆(3) staining populations 72 h after addition of OHT. The data for OHT alone and OHT plus 25 μ M Z-VAD (–1h) are from 3 independent experiments, and the difference between apoptosis in the cells treated with OHT alone and in cells maintained in OHT + Z-VAD is significant at (**, P 0.01).

FIG. 2.

Caspase 9 and caspase 3 are activated in OHT-ER induced apoptosis. Cells were maintained in medium containing either the ethanol vehicle or 10 nM OHT, incubated for 72 hours and stained with the caspase 3 (panels A and B) or caspase 9 (panels C and D) fluorescent peptides. APO LOGIX Carboxyfluorescein Caspase Detection Kits from Cell Technology were used. The labeled peptide FAM-DEVD-FMK was used for caspase-3 and FAM-LEHD-FMK was used for caspase-9. Assays were carried out according to the supplier's protocol. The data is representative of 3 independent experiments.

FIG. 3.

Knockdown of p38α expression moderately reduces OHT-ER-induced apoptosis. A. The cells were treated with 100 nM OHT for the indicated times, or with 12.5 μM etoposide (Etop) for 24 h. Whole cell lysates were prepared and analyzed by Western blotting for phosphorylated p38 and for total p38 protein. B. Whole cell lysates were prepared from non-transfected cells (None), from mock-transfected cells (Mock), or from cells transfected with either the p38α siRNA (p38), or a control siRNA (pGL3, luciferase). Western blotting was used to analyze p38α expression. Actin was used as a loading control. The data in panels A and B is representative of multiple experiments. C. Band intensity of Western blots was quantified using a Storm PhosphorImager. The data represents the average ratio (p38/pGL3) of band intensity ± SEM of the p38α and actin bands from three Western blots. The difference in p38α levels between the control pGL3 siRNA and the p38α siRNA is significant (*, P = 0.05, n = 3). D. The cells were transfected with either the p38α or pGL3 siRNA. After 48 hours, 100 nM OHT was added to some cells and cell death was analyzed 3 days later using DiOC₆(3) staining followed by flow cytometry as described in the Experimental Procedures. The data in panel D is the average of 4 independent experiments.

FIG. 4.

Inhibition of p38 phosphorylation partially reduces OHT-ER-induced cell death. A. Cells were pretreated with 5 μ M SB203580 (SB) for 1h before the addition of 100 nM OHT. Cell lysates were prepared after incubation for 24 and 48h and were analyzed for phosphorylated p38 by Western blot. Lysates prepared from cells not treated with OHT or SB were used as background controls. B. The cells were transfected with either the p38 α or pGL3 siRNA. After 48 hours, SB (5 μ M) was added to some cells 1 hour before addition of 100 nM OHT. Programmed cell death was analyzed by flow cytometry as indicated by reduced DiOC₆(3) staining.

FIG. 5.

Inhibiting JNK activity reduces OHT-ER-induced cell death. A. The cells were treated with 100 nM OHT for the indicated times (hours) and phosphorylated JNK was determined by Western blotting. The same membrane was stripped and re-blotted with an anti-actin antibody to show equal protein loading. B. The cells were pretreated with 25 μ M SP600125 (SP) for 1h followed by the addition of OHT. After 3 days, DiOC₆(3) staining followed by flow cytometry was used to evaluate early stage apoptosis. The data represents the average of 4 experiments $\pm$ sem. C. Cells were pretreated with 25 μ M SP for 1h before the addition of 100 nM OHT. Cell lysates were prepared after 48h of incubation and were analyzed for phosphorylated JNK by Western blot. Lysates prepared from cells not treated with OHT or SP were used as background controls. Calnexin in cell lysates was detected for equal loading of proteins.

FIG. 6.

The ERK inhibitor PD98059 blocks OHT-ER-induced cell death. A. The cells were pre-incubated for one hour with DMSO, or with PD98059 at 2, 10 and 50 μ M, and then treated with 100 nM OHT (+OHT, black bars), or with the ethanol vehicle (–OHT, open bars), for 3 days. Cell death was analyzed by measuring PI-positive cell populations by flow cytometry as described in Experimental Procedures. B. The cells were pre-treated with 50 μ M PD98059 (+PD), DMSO vehicle (–PD) or 10 nM ICI 182,780 for one hour. Cell death was induced by adding: 12.5 μ M etoposide (Etop), or 1 μ M tamoxifen (Tam), or 100 nM OHT, or 20 μ M OHT for 72 h. To evaluate the protective effects of PD98059 or ICI 182,780, PI-positive cell populations were measured by flow cytometry. Experiments comparing apoptosis in cells treated with 100 nM OHT to apoptosis in cells treated with 100 nM OHT and 50 μ M PD were performed 3 times $\pm$ sem (**, two-sided $P=0.004$) The error bars for ICI are too small to see. The other treatments were repeated at least twice.

FIG. 7.

OHT induces delayed and persistent activation of ERK1/2, which is blocked by PD98059.

A. The cells were treated with either 100 nM OHT, or with 10 nM E₂, with or without pre-incubation with 50 μ M PD98059 for 1 h. Control cells were incubated in medium containing 0.2% DMSO or 0.1% ethanol (EtOH) as vehicle controls. Whole cell lysates were prepared at the indicated times after the addition of OHT and E₂. The samples were subjected to Western blot analysis and probed with a phospho-ERK1/2 antibody (p-ERK antibody that is specific for phosphorylation of Thr202.Tyr 204). The membranes were then stripped and re-probed for total ERK1/2 protein (ERK). The data in panel A is representative of many related experiments. B. 50 μ M PD98059 was added to the cells an hour before (-1 h), at the same time (0 h), or 1, 4, 12, 24 and 48 h after treatment with 100 nM OHT. Cells were harvested, stained with PI and analyzed by flow cytometry at 86 hours.

FIG. 8.

EGF- α enhances ER phosphorylation and OHT-ER-induced apoptosis. EGF- α (100 ng/ml) was added to the medium either 1 h before (-1h) or 24 after (24h) addition of 100 nM OHT. Apoptosis was analyzed by DiOC₃(6) staining followed by flow cytometry. Data were the mean of three independent experiments $\pm$ sem with $P < 0.05$ for OHT alone compared to OHT plus EGF (OHT/EGF -1h).

Fig. 9.

ICI 182,780 blocks the persistent activation of ERK1/2 by E₂ and by OHT. The cells were pre-incubated with, or without, 1000 nM ICI 182,780 for 1 hour before addition of 100 nM OHT or 10 nM E₂ with Ethanol (EtOH) as control. The cells were harvested after 12, 24 and 48 hours and phospho-ERK1/2 and total ERK1/2 was detected by Western blotting using antibodies specific for phospho-ERK1/2 and ERK1/2.