Tamoxifen Induction of CCAAT Enhancer-binding Protein α Is Required for Tamoxifen-induced Apoptosis*

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Low concentrations of tamoxifen or its active metabolite 4-hydroxytamoxifen (OHT) induce estrogen receptor α (ERα)-dependent apoptosis. To analyze the pathway of OHT-ERα-induced apoptosis, we developed stably transfected lines of HeLa cells expressing wild-type ER and an inactive mutant ERα unable to bind estrogen response elements. HeLa cells expressing the mutant ERα and HeLa cells expressing wild-type ERα in which the ER was knocked down with an ER-specific small interfering RNA were not killed by Tam or OHT, suggesting that estrogen response element-mediated transcription is required for Tam- and OHT-induced apoptosis. Microarray analysis to identify a gene(s) whose expression is important in OHT-ERα-mediated apoptosis identified 19 mRNAs that OHT up-regulated by >1.6-fold and 15 down-regulated mRNAs. Gene function and the time course of induction by OHT-ERα led us to further investigate CCAAT enhancer-binding protein α (C/EBPα), which has roles in cell cycle progression and apoptosis, and p21. Quantitative reverse transcription-PCR, Western blot analysis, and RNA interference knockdown suggest that cell cycle arrest resulting from OHT-ERα induction of p21 may facilitate apoptosis. OHT-ERα, but not E2-ERα, induced C/EBPα mRNA and protein. RNA interference knockdown of C/EBPα nearly abolished OHT-ERα-induced apoptosis. We isolated stable cell lines that were resistant to OHT-induced apoptosis, contain full-length functional ERα, and undergo apoptosis in response to etoposide. In these OHT-resistant cell lines both before and after OHT treatment, C/EBPα levels are much lower than in OHT-sensitive cells. These studies establish a novel molecular site responsible for Tam- and OHT-ERα-induced apoptosis of cancer cells.

Estrogens, acting through estrogen receptor α (ERα)3 and ERβ, exert pleiotropic effects on diverse cells and organ systems. Binding of a potent estrogen such as 17β-estradiol (E2) to the ER may induce dissociation of ER from a heat shock protein/chaperone complex (1) and enable the ER to dimerize and bind to specific DNA sequences termed estrogen response elements (EREs) (2). Transcription activation by ERs is mediated by two interacting activation functions, AF1 and AF2. AF1 mediates ligand-independent transactivation and is thought to be especially important in transcription by the clinically important selective estrogen receptor modulator, tamoxifen (Tam), and by its active metabolite, 4-hydroxytamoxifen (OHT). AF2 activity depends on estrogen binding to the ER ligand binding domain (LBD) (3–7). When bound to estrogen, the ER LBD acquires a conformation that enables the recruitment of coactivators. The bound coactivators help assemble a multiprotein complex that facilitates both chromatin remodeling and formation of an active transcription complex (8). When bound to Tam or OHT, the ligand binding domain of ER assumes a different conformation that interferes with coactivator binding and appears to facilitate corepressor binding (9).

By competing with E2 and other estrogens for binding to the ligand binding site of ER, 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. Tam and OHT can both arrest cell growth by preventing the growth promoting activities of E2 and can induce death of both ER-positive and ER-negative cells (10–12). Tam triggers apoptosis in vitro and shrinks some tumors in vivo. Several mechanisms have been proposed to explain tamoxifen-induced cell death including transcriptional regulation of Bcl-2 family proteins (13), activating mitogen-activated protein kinase and other kinases through nongenomic pathways (11), and triggering an increase of intracellular Ca2+ (14). However, in many of these experiments, very high μM 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 were otherwise identical and differed 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 (HeLaER6 (15)). We identified two pathways by which OHT induces cell death. When ER-negative HeLa cells are maintained in medium containing 10–20 μM Tam, OHT, E2, or raloxifene, the cells die within 24 h by a reactive oxygen-based pathway that triggers classical caspase-dependent apoptosis (16). Low (nm) concentrations of OHT
We analyzed the roles of C/EBPα RNAi and stable transfection of mutant ERs to examine the transcription in OHT-ER-induced cell death. We initially used cells containing ERα classical reactive oxygen-based, caspase-3-dependent, death pathway. In enhancer-binding protein mediated gene transcription in cell death, we carried out microarray with p21 and cyclin-dependent kinase have been proposed on protein-protein interactions through C-terminal interaction.

In this study we analyzed the role of ERα-mediated gene transcription in OHT-ER-induced cell death. We initially used RNAi and stable transfection of mutant ERs to examine the requirement for ERα and for ERα-mediated gene transcription. Because these experiments supported a role for OHT-ERα-mediated gene transcription in cell death, we carried out microarray analysis.

The microarray data, more detailed regulatory studies, and what was known about their function led us to focus on CCAAT enhancer-binding protein α (C/EBPα) and p21/waf1/Cip1/Cip1. C/EBPα and p21 encoded proteins whose roles in cell growth and cell death and expression patterns were consistent with a possible role in OHT-ERα-dependent apoptosis.

C/EBPα by OHT-ER plays the central role in OHT-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids pIE-LBD and pIE-M-DBD**—Restriction enzymes were obtained from Promega. Plasmid pIE-LBD was constructed by subcloning the EcoRI/BamHI fragment of pCMV-hER(ER) (30) into pRESIneo (Clontech, Palo Alto, CA). Plasmid pIE-M-DBD was constructed by using QuikChange site-directed mutagenesis (Stratagene, San Diego, CA) with primers 5'-GGAGTCTGGTCTCTGTGGGCTTGCAAGG CCCCTTCTCAAG-3' and 5'-CTTGAAGAAGCCCTTG CAGGGCCCCAGGACGACTCC-3' on pCMV-HER. This results in the mutations E203A and G204A in ERα. The EcoRI/BamHI fragment was subcloned into pRESIneo.

**Cell Culture**—HeLaER cells were grown in Phenol Red-free Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% charcoal-dextran-treated fetal bovine serum and 150 μg/ml G418 (Invitrogen).

**Establishment of the HeLaER LBD and HeLaER M-DBD Cell Lines**—HeLa cells were plated in 100-mm culture dishes at a concentration of 1.6 × 10^6 cells per dish. HeLa cells obtained from ATCC were used to construct the HeLaER M-DBD and HeLaER LBD cell lines. The original ERα M-DBD mutant was a gift from Prof. L. Jameson and was recloned into the pIE plasmid (15). The cells were transfected with 6 μg of Sspl-linearized pIE-LBD, pIE-M-DBD using Polyfect (Qiagen, Valencia, CA) lipidosome following the manufacturer’s instructions. The medium was replaced with selection media (50% Dulbecco’s modified Eagle’s medium, 10% charcoal-dextran-fetal bovine serum, 50% conditioned medium, 500 μg/ml G418 in the HeLaER LBD and HeLaER M-DBD selections) ~24 h post-transfection. After sufficient growth, colonies were isolated and reseeded into 24-well culture plates in selection medium. Cell lines were further expanded to confluence in 6-well plates, then T-75 flasks. For long-term growth, the cells were maintained under selection in medium containing 150~200 μg/ml G418. ER expression was detected by Western blotting. H222 anti-ER antibody (NeoMarkers) was used to detect ER in the HeLaER LBD cells, and NCL-ER-6F11 anti-ER antibody was used to detect ER in the HeLaER M-DBD cells.

**Isolation of OHT-resistant Cell Lines**—HeLaER cells in Phenol Red-free Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% charcoal-dextran-treated fetal bovine serum and 150 μg/ml G418 were plated in 96-well plates at 0.5 cell/well in medium containing 100 nM OHT. After several weeks, colonies from wells containing a single colony were isolated as OHT-resistant clonal cell lines.

**Microarray Analysis**—HeLaER cells were treated with 10 nM OHT or ethanol vehicle for 34 h. RNAs were purified with the RNeasy® kit (Qiagen). The mRNAs were reverse-transcribed to cDNAs by using Moloney murine leukemia reverse transcriptase (Invitrogen) and hybridized to Affymetrix GeneChip® Human Genome U133A Arrays (Affymetrix, Santa Clara, CA). The array data were initially processed by the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois using the GeneChip Robust Multichip Average package in R/Bioconductor. A false discovery rate of <0.05, as deter-
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Of the 2083–2103 of transcript variants 1 and 2, respectively. One µg of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen), and 1% of the cDNA product was used in quantitative RT-PCR. The forward and reverse primer mix (1 µl of 10 µM) and 12.5 µl of SYBR® green 2 × PCR master mix (Applied Biosystems, Carlsbad, CA) in a total volume of 25 µl were put into each well of a 96-well iCycler iQ™ PCR plate and assayed using a Bio-Rad iCycler™ optical system (Bio-Rad). PCR was as follows: 95 °C for 2 min; 45 repeats (95 °C for 25 s, 55 °C for 25 s, 72 °C for 15 s); melting from 55 to 95 °C with 0.5 °C increases per 10 s in each cycle for 80 cycles. The internal standard was 36B4 mRNA.

RNA Interference—siRNA sequences were BLAST-searched against the human genome to ensure that they were sequence-specific for ERα, C/EBPα, and p21. The siRNA sequences showed no exact or near exact matches to any other sequences in the human genome. siRNAs were synthesized by Dharmacon (Chicago, IL). ERα siRNA: 5'-AAGCUACUGUUUGCUCCUAACTT-3' (nucleotides 1201–1223 of hERα relative to the first nucleotide of the start codon); C/EBPα siRNA: 5'-AGC AGA CGU CCA UCG ACA U-3' (nucleotides 173–191); p21 siRNA: 5'-CAU ACU GCC CUG GAC UGU UU-3' (nucleotides 1943–1963 or 2083–2103 of transcript variants 1 and 2, respectively. Variant 2 contains an extra internal sequence segment in the 5'-untranslated region but encodes the same protein as Variant 1.). When cells were ~30–40% confluent, 4 µl of the 20 µM siRNAs were transfected into the HeLaER cells in 12-well plates with Oligofectamine™ reagent (Invitrogen).

Western Blotting—Cells were resuspended in ice-cold lysis buffer (adapted from Schreiber et al. (32)): 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 10 mM dithiothreitol, 100 µg/ml phenylmethylsulfonyl fluoride, protease inhibitor mixture (P8340, Sigma) was added right before use. After sonication on ice, the extracts were centrifuged for 5 min at 4 °C, and protein concentration was determined with Coomassie Blue (Bio Rad). Samples were combined with 6× SDS loading buffer, boiled for 5 min, and loaded on to a 12.5% SDS-polyacrylamide gel. After electrophoresis, the gel samples were transferred to nitrocellulose membranes (Schleicher & Schuell). Primary and secondary antibodies were each used at a 1:1000 dilution. Primary antibodies were C/EBPα (sc-9314), pc21 (sc-6246), c-Myc (sc-40), phosphorylated extracellular signal-regulated kinase, actin, and calnexin (Santa Cruz Biotechnology, Santa Cruz, CA) and estrogen receptor α antibody (Clone 6F11, Biocare Medical, Walnut Creek, CA). Proteins were visualized by reacting with ECL plus reagents (Amer sham Biosciences), and band intensity was quantitated using a GE Healthcare Storm PhosphorImager (Amersham Biosciences).

RESULTS

RNAi Knockdown of ERα Blocks OHT-mediated Apoptosis—Our observations that low concentrations of Tam and OHT do not induce apoptosis of wild-type ER-negative HeLa cells and that pretreatment with an excess of ICI 182,780/Faslodex (ICI), the selective estrogen receptor modulator raloxifene (RAL), or E2 largely protected HeLaER cells from OHT- and TAM-induced death (16) strongly suggested a role for ERα in Tam- and OHT-induced cell death. To test the role of ERα more directly, we carried out an RNAi knockdown of ERα in the HeLaER cells. The cells were transfected with either the control pGL3 (luciferase) siRNA or the ER-specific siRNA. Western blot analysis showed that the ERα knockdown lasted at least 5 days (Fig. 2A). The cells were transfected with the control pGL3 siRNA or with the ERα siRNA and then treated with either EtOH vehicle or 10−8 M OHT (Fig. 2B). Cells transfected with the pGL3 siRNA and treated with OHT became sparse and exhibited the elongated morphology we previously described (16). When displayed using fluorescence-activated cell sorting, the OHT-treated cells transfected with the pGL3 siRNA exhibited a shoulder showing a substantial population of cells undergoing apoptosis. In contrast, in the cells in which ERα was knocked down with the ERα-specific siRNA, OHT did not cause a reduction in cell number, a change in cell morphology, or the appear-
ance of a peak of apoptotic cells (Fig. 2B). These data clearly demonstrate that ERs mediate OHT- and TAM-induced cell death in HeLaER cells.

OHT Does Not Induce Apoptosis in Stably Transfected Cell Lines Expressing ER Mutants That Do Not Bind to EREs—Although our data show that ER is required for OHT-induced cell death, the functions of ER that are essential were not known. ERα exerts its actions by rapid nongenomic actions based on modulation of signal transduction pathways (33–38) and direct binding to DNA at palindromic EREs and dispersed ERE half-sites (45). To identify the ER functions important in OHT–ERα-induced apoptosis, we isolated stably transfected lines of HeLa cells expressing mutant ERs defective in specific ER functions. Because the ER LBD alone reportedly is sufficient for many non-genomic actions of ER, we isolated a stably transfected cell line expressing only the ERα LBD that cannot activate transcription by either direct binding to DNA or through tethering, and the ERα M-DBD that only activates transcription through tethering. Very high, >10 μM, OHT should induce apoptosis through the ER-independent pathway (Fig. 1). Because all three cell lines were killed by 20 μM OHT, they remained sensitive to caspase-dependent apoptosis (Fig. 2, 2 × 10⁻⁵ M). OHT at 10–1000 nM should induce apoptosis through the ER-dependent pathway (Fig. 1). The HeLaER, HeLaER LBD, and HeLaER M-DBD cell lines were maintained in medium containing either ethanol vehicle or the indicated concentrations of OHT (10 nM to 20 μM) for 3 days, and cell death was determined (16). As expected, OHT at 10–1000 nM induced apoptosis in the HeLaER cells. Neither the HeLaER LBD nor the HeLaER M-DBD cell lines were killed by 10–1000 nM OHT (Fig. 3). Similar results were obtained with Tam (data not shown). These data support the idea that ERα-mediated transcription is required for OHT–ERα-induced apoptosis.

ICI Protects HeLaER Cells from OHT-induced Cell Death up to 33 h Post-OHT Treatment—We previously reported that ICI 182,780 does not induce apoptosis. By competing with OHT for binding to ERα, the addition of excess ICI 182,780 blocks OHT–ERα-induced apoptosis (16). To determine whether ICI can protect HeLaER6 cells when it is added to the medium after OHT, HeLaER6 cells were treated with 10⁻⁸ M OHT and 10⁻⁸ M ICI 182,780 0, 2, 12, 24, 33, 36, and 48 h after OHT treatment. Consistent with our earlier report, the addition of an excess of ICI 182,780 at the same time as OHT blocked OHT-induced apoptosis. Surprisingly, the addition of ICI 182,780 up to 33 h after OHT also blocked OHT-induced apoptosis (Fig. 4). By 36 h the cells commit to OHT–ERα-induced apoptosis, and ICI begins to lose its ability to block OHT–ERα-dependent activation of an AP1-luciferase plasmid as previously reported (data not shown and Ref. 47). To examine the role of ER-mediated transcription in OHT–ERα-induced apoptosis, we examined at the ability of OHT to induce apoptosis of the cell lines expressing wild-type ERα, the ERα LBD that cannot activate transcription by either direct binding to DNA or through tethering, and the ERα M-DBD that only activates transcription through tethering. Very high, >10 μM, OHT should induce apoptosis through the ER-independent pathway (Fig. 1). Because all three cell lines were killed by 20 μM OHT, they remained sensitive to caspase-dependent apoptosis (Fig. 3, 2 × 10⁻⁵ M). OHT at 10–1000 nM should induce apoptosis through the ER-dependent pathway (Fig. 1). The HeLaER, HeLaER LBD, and HeLaER M-DBD cell lines were maintained in medium containing either ethanol vehicle or the indicated concentrations of OHT (10 nM to 20 μM) for 3 days, and cell death was determined (16). As expected, OHT at 10–1000 nM induced apoptosis in the HeLaER cells. Neither the HeLaER LBD nor the HeLaER M-DBD cell lines were killed by 10–1000 nM OHT (Fig. 3). Similar results were obtained with Tam (data not shown). These data support the idea that ERα-mediated transcription is required for OHT–ERα-induced apoptosis.
induced apoptosis (Fig. 4). These data suggest that OHT-ERα mediates a key step in activation of the death pathway 33–36 h after OHT treatment.

Because our data indicated that ERE-mediated transcription was required for OHT-ERα-induced apoptosis, we carried out microarray analysis to identify mRNAs whose expression is regulated by OHT-ERα. We then analyzed likely mRNAs identified from the microarray analysis to determine whether or not they play a role in OHT-mediated apoptosis. Because the ICI data suggested that a key event in committing the cells to OHT-mediated apoptosis occurs in the 33–36-h time frame, we looked at mRNA levels 34 h after OHT treatment.

Identification of OHT-ERα-regulated Genes by Microarray Analysis—Microarray studies in ERα positive MCF-7 cells and in MDA-MB-231 breast cancer cells stably transfected to express ERα indicated that Tam and OHT act as full or partial agonists on ~20% of E2-regulated genes and also regulate a group of genes not regulated by E2-ER (48). We, therefore, carried out microarray analysis to identify OHT-ERα-regulated genes. HeLa-ER cells were maintained in medium containing 10 nm OHT for 34 h, the cells were harvested, and RNA was isolated, converted to cDNA, and analyzed on Affymetrix microarrays containing 18,000 human genes. We focused on the 19 genes whose mRNAs were up-regulated by >1.6-fold after OHT treatment and the 15 mRNAs whose levels were down-regulated by >1.5-fold after OHT treatment (Table 1). Approximately 50 additional mRNAs were up-regulated 1.5–1.6-fold after OHT treatment (data not shown). Because none of these mRNAs encoded proteins that seemed likely to mediate OHT-dependent apoptosis, the regulation of these mRNAs was not further characterized. The genes whose mRNA levels were regulated by OHT included two genes (C/EBPα and p21/Cdkn1a/waf1/Cip1) that code for proteins likely to be important in cell growth or cell death (Table 1). C/EBPα is a transcription factor implicated in cell death in terminally differentiated cells, and p21 is a widely studied cyclin-dependent kinase inhibitor (CDKNA1/waf1/Cip1).

OHT-ER Induction of C/EBPα and p21 mRNAs—We used quantitative RT-PCR to confirm the microarray data and to assay mRNA levels at selected time points. Consistent with other data using this system, in most cases the -fold induction was greater by quantitative RT-PCR than by microarray (48).

Because E2 does not induce cell death, we concentrated on mRNAs that were regulated differently by OHT and by E2. Quantitative RT-PCR showed that p21 mRNA is induced by both OHT and by E2 in the 24–48 h time frame important for OHT-induced apoptosis (Fig. 5A) and shows little or no induction by RAL and ICI 182,780. C/EBPα mRNA is strongly induced by OHT, is not induced by E2, and exhibits very weak induction by ICI 182,780 (Fig. 5B). We also tested the effects of E2 and OHT on the levels of C/EBPα and p21 protein. Western blot analysis shows that E2 does not induce C/EBPα protein (Fig. 5C, C/EBPα, E2OHT, and E2) and moderately induces p21 (Fig. 5C, p21, E2OHT, and E2), whereas OHT strongly induces both C/EBPα and p21 (Fig. 5C). The role of C/EBPα in apoptosis in other systems (29), the strong induction of C/EBPα mRNA in the important time period 24–48 h after OHT treatment (Fig. 4), and the induction of C/EBPα mRNA by OHT, which induces apoptosis of the HeLaER cells, and the absence of a strong induction of C/EBPα by E2 or ICI, which do not induce apoptosis, all are consistent with the idea that OHT induction of C/EBPα may be important in OHT-ER induced apoptosis.

TABLE 1

| Genes whose expression is regulated by OHT in HeLaER cells |
|-------------------------------------------------------------|
| OHT (10 nm) was added at 0 time, the cells were harvested at 34 h, and mRNA was isolated and analyzed using microarrays as described under “Experimental Procedures.” The lane labeled OHT shows mRNAs induced by OHT at least 1.6 fold (up-regulated genes) or down-regulated (down-regulated genes) by at least 1.5-fold. The first row shows the -fold change as seen on the microarray. The second row represents the -fold change of most of the genes as shown by quantitative RT-PCR. A few genes whose expression is unlikely to be related to cell death (such as complement C3) were not further analyzed. Regulation of the expression of some mRNAs by 10 nm E2 and by 10 nm OHT at various times is shown for some mRNAs that were studied in more detail. TFF, transforming growth factor. |

| mRNA   | Fold change of up-regulated genes | Fold change of down-regulated genes |
|--------|----------------------------------|-----------------------------------|
|        | Array 34 h OHT  | RT-PCR, 24–34 h E2     | |
|        | OHT | E2          | OHT                      | E2 | |
| TFF2   | 3.4 | 106.8 | 92 | |
| KT19   | 2.37 | 2.56 | 5.66 | |
| TMOD1  | 2.36 | 6.7 | 6.5 | |
| PS2    | 2.33 | 6 | 33.5 | |
| p21    | 2.28 | 5.5 | 5 | |
| PDZK1  | 2.14 | 5 | 5 | |
| SNAI2  | 2.13 | 4.7 | 2 | |
| KRT13  | 2.08 | 4.7 | 2 | |
| TGFα   | 1.97 | 7.7 | 7.07 | |
| C/EBPα | 1.84 | 12 | 2 | |
| C3     | 1.84 | 12 | 2 | |
| TPM1   | 1.81 | 2.4 | 2 | |
| ABCB1  | 1.72 | 4.7 | 2 | |
| IEX-1  | 1.72 | 2.1 | 3.2 | |
| UNC119 | 1.7 | 4.97 | 2 | |
| FOSL2  | 1.64 | 2.38 | 2.24 | |
| RGS19  | 1.64 | 5.42 | 2.24 | |
| DUSP9  | 1.62 | 2.41 | 2 | |
| IPT1R  | 1.6 | 2.74 | 2 | |

FIGURE 4. ICI 182,780, added up to 33 h after OHT prevents OHT-ERα-induced apoptosis. OHT (10 nm) was added to the medium at 0 h. At the indicated times after the addition of OHT, 1 µM ICI 182,780 was added. Cell death was assayed as described under “Experimental Procedures” at 72 h after the addition of OHT. The data represent the average ± S.E. for three separate experiments.
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In Cells Resistant to OHT-ERα-induced Apoptosis, Expression of C/EBPα and OHT Induction of C/EBPα Are Lost—The expression and RNAi knockdown data strongly supported a role for C/EBPα in OHT-ERα-induced cell death and suggested a possible role for p21. If OHT induction of C/EBPα or p21 plays a key role in OHT-ERα-induced apoptosis, HeLaER cells selected for resistance to OHT-ERα-induced cell death might show altered expression or regulation of C/EBPα or p21. The overwhelming majority of HeLaER cells stop growing and die in medium containing 100 nM OHT, but a few cells survive. Based on the RNAi knockdown experiment (Fig. 2), we knew that HeLaER cells that lose ERα expression would continue to grow in OHT and would be represented among the clonal lines we isolated. Western blot analysis showed that half of the clonal lines we isolated after growth in OHT-containing medium had lost all or most of their ERα expression or an ERα fragment (data not shown). We identified four clonal cell lines that were resistant to OHT-ERα-induced apoptosis and contained levels of full-length ERα comparable with two HeLaER cell lines that were sensitive to OHT-ERα-induced apoptosis (Fig. 8A). Data for all of the ERα-containing, OHT-resistant clonal cell lines we isolated are shown. No cell lines were excluded. Etoposide, a widely used activator of caspase-dependent apoptosis, retained the ability to kill all 4 of the OHT-resistant cell lines in 24 h (data not shown). Therefore, the four OHT-resistant cell lines are specifically defective in OHT-induced apoptosis but retain functional machinery for caspase-dependent apoptosis activated by the mitochondrial pathway.

We used quantitative RT-PCR to determine the basal levels of C/EBPα and p21 in the OHT-sensitive and OHT-resistant cell lines and their regulation by OHT and by E2. The basal level of p21 mRNA in the OHT-resistant cell lines was equal to or plasma membrane integrity, and light scattering detects changes in cell morphology. In preliminary studies, we identified a time after OHT addition that resulted in substantial cell death as measured by all three assays and showed that transfecting the C/EBPα and p21 siRNAs into the cells using oligofectamine did not cause cell death (data not shown). In three independent experiments the addition of OHT to the medium induced cell death as measured by all three assays (Fig. 7A, OHT). Transfection with the control pGL3 luciferase siRNA had no effect on cell death (Fig. 7A, PGL3 siRNA + OHT). RNAi knockdown of C/EBPα nearly abolished OHT-ERα-induced cell death (Fig. 7A, CEBPα siRNA + OHT). The difference between the level of cell death in the cells treated with OHT or treated with OHT and transfected with the pGL3 siRNA compared with the level of cell death in the cells transfected with the C/EBPα siRNA was significant using each of the three assays for cell death (p < 0.05). RNAi knockdown of p21 had a significant but smaller effect on OHT-ERα-induced cell death (Fig. 7B). There was a statistically significant (p < 0.05) decrease in cell death in the cells treated with the p21 siRNA compared with the cells transfected with the pGL3 siRNA. A second control siRNA recommended by the supplier (Dharmacon) also had no effect on OHT-ERα-induced cell death (Fig. 7B). These data show that induction of C/EBPα by OHT plays a key role in the ability of OHT to induce apoptosis.

Regulation of C/EBPα and p21 Protein Levels and Their Knockdown Using RNAi—Because we were going to test the roles of p21 and of C/EBPα in OHT-ERα-induced apoptosis using RNAi knockdown and we wanted to determine whether OHT and E2 induce p21 and C/EBPα proteins, we carried out Western blot analysis. OHT induced p21 protein to a level higher than was seen with E2 (Fig. 6). As expected, OHT, but not E2, induced C/EBPα protein. C/EBPα was knocked down by the C/EBPα siRNA and not by the p21 siRNA. Similarly, p21 was knocked down by the p21 siRNA but not by the C/EBPα siRNA (Fig. 6).

RNAi Knockdown of C/EBPα Blocks OHT-ERα-induced Apoptosis—We then looked at the effect of knockdown of C/EBPα on apoptosis. To evaluate cell death, we used three fluorescence-activated cell sorter-based assays. These assays target distinct aspects of cell death. Release of a fluorescent dye evaluates loss of mitochondrial membrane potential, uptake of a fluorescent agent that intercalates into DNA targets the loss of

![Figure 5. Time course of regulation of C/EBPα and p21 mRNA levels by ER ligands. A and B, the cells were maintained in medium containing ethanol vehicle (EtOH), 10 nM OHT, E2, RAL, or ClI 182,780 for the indicated times, and RNA was isolated and analyzed by quantitative RT-PCR as described under "Experimental Procedures" for the relative abundance of p21 mRNA (A) and C/EBPα mRNA (B). The level of each mRNA in the ethanol vehicle sample was set equal to 1. C, Western blot analysis of the induction of C/EBPα and p21 after 48 h in 10 nM E2 or 10 nM OHT. Calnexin was used as the internal standard.]

![Figure 6. OHT-induction and RNAi knockdown of C/EBPα and P21 proteins. Western blot analysis in untransfected cells and in cells transfected with C/EBPα-specific siRNA (5′-ACGACAGCUCCAGUCAUA-3′), p21-specific siRNA (5′-CAUACUGGCCUCUGACUUU-3′), or control pGL3 (luciferase) siRNA (59). Actin was used as the internal standard.]

![Image 285x26 to 306x38]
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DISCUSSION

At least three lines of investigation demonstrate that OHT acts by binding to ERα to induce apoptosis. Wild-type HeLa cells which lack ER and HeLa cells that contain ER mutants are not killed by 10–1000 nM OHT (Ref. 16 and Fig. 3). High concentrations E2, ICI 182,78, and RAL block OHT-induced apoptosis (16). RNAi knockdown of ERα abolishes OHT-induced cell death (Fig. 2). Therefore, loss of expression of ERα or mutational inactivation of ERα are likely to be observed in some cell lines resistant to OHT-induced cell death. Consistent with a key role for ERα in OHT-induced cell death, in several cell lines resistant to OHT-induced cell death we detected loss of ERα expression or production of a truncated ERα (data not shown).

A central role for C/EBPα in OHT-ERα-induced apoptosis is supported by our observations that RNAi knockdown of C/EBPα nearly abolishes OHT-ERα-induced apoptosis and by the loss of C/EBPα and of OHT induction of C/EBPα in cell lines selected by their ability to grow in concentrations of OHT that kill nearly all of the HeLaER cells. It is striking that all of the cell lines that we isolated that grew out in 100 nM OHT either exhibited reduced expression of full-length ERα or reduced expression and defective regulation of C/EBPα.

Because reduced expression of C/EBPα nearly abolished OHT-induced apoptosis, it seemed likely that expression of increased levels of C/EBPα enhances OHT-induced cell death. To test this idea, we attempted to transiently transfect wild-type C/EBPα and C/EBPα mutants defective in specific functions (28) into the HeLaER cells. However, we were unable to transfec more than ~30% of the cells, and the harsh conditions needed for high transfection efficiency damaged the cells. It was, therefore, not possible to rapidly test the effect of elevated levels of C/EBPα on OHT-mediated apoptosis.

C/EBPα is expressed in many cell types including breast and ovary tissues and localizes to the nucleus. C/EBPα mRNA gives rise to two isoforms of protein, the full-length 42-kDa and lines resistant to OHT-induced apoptosis provides compelling support for a central role of C/EBPα in cell death mediated by OHT-ERα.
30-kDa with a truncated N terminus. The two isoforms of C/EBPα show contrasting functions in gene activation and cell proliferation, and the isoform ratio may be important in mediating proliferation and differentiation (49). Our Western blot data indicate that nearly all of the C/EBPα in these cells is the 42-kDa isoform, and this isoform is responsible for OHT-ERα-induced apoptosis. Many studies of C/EBPα focus on its role in lineage determination in hematopoiesis (50). C/EBPα is a key factor in driving the development of myeloid cells. Our data suggest a quite new but plausible role of C/EBPα as a critical factor in controlling an apoptosis pathway. Our finding of a role for C/EBPα in cell death, consistent with several reports describing diverse pathways including p21, and the E2F complex that are impacted by C/EBPα as it mediates cell cycle arrest and proliferation (51–53). Induction of C/EBPα expression in breast cancer cells resulted in growth inhibition accompanied by G0-G1 cell cycle arrest. C/EBPα expression was associated with up-regulation of p21 and peroxisome proliferator-activated receptor γ and down-regulation of c-Myc (52). We suggest that p21 works with C/EBPα to help determine whether or not cells initiate apoptosis after cell cycle arrest.

Both C/EBPα and p21 are induced by OHT-ERα, and it is likely that the interplay between these two proteins plays a role in OHT-ERα-induced cell death. Although both E2 and OHT induce p21 mRNA in HeLaER cells, induction of p21 protein by OHT is far more effective than induction of p21 by E2. The cyclin-dependent kinase inhibitor p21 exhibits a short half-life of 0.5 to <2 h (54, 55) and is thought to be stabilized by binding to C/EBPα (56). Because OHT, but not E2, induces C/EBPα, which stabilizes the p21 protein, OHT elicits a much larger induction of p21 protein than E2.

RNAi knockdown of p21 resulted in a moderate reduction in OHT-induced apoptosis. It is possible that the failure to isolate OHT-resistant cell lines that lack p21 stems from an essential role of p21 in regulating progression through the cell cycle.

Because p21 is a well known cyclin-dependent kinase inhibitor and participates in regulating the cell cycle and C/EBPα reportedly inhibits progression through the cell cycle (23–27) and contains binding sites for p21 and cyclin-dependent kinase (28), it is quite possible that OHT-induced apoptosis may require C/EBPα to associate with p21 and initiate downstream pathways leading to cell death. This might involve arresting cell growth. Consistent with this view, in MCF-7 cells tamoxifen acts mainly by arresting cells in the G0/G1 phase of the cell cycle (57, 58). Of course the ability of C/EBPα to mediate gene expression by acting as a transcriptional factor or to play a role in signal transduction pathway in the cytoplasm may also be important to its role in OHT-induced cell death.

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