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Proteasome-independent down-regulation of estrogen receptor-α (ERα) in breast cancer cells treated with 4,4′-dihydroxy-*trans*-stilbene

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ABSTRACT

Treatment of cells with estrogens and several pure ERα antagonists rapidly induces down-regulation of the α-type estrogen receptor (ERα) in the nucleus by mechanisms that are sensitive to the proteasome inhibitors, MG132 and *clasto*-lactacystin-β-lactone. Hence, it is believed that these ER ligands induce down-regulation of ERα by proteasome-dependent mechanisms, which serve to control both the amount of transcriptional activity and the level of ligand-bound ERα in cells. In this study, we observed that treatment of cultured MCF-7 and T47D human breast cancer cells with the low affinity ER ligand, 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), inhibited the transcriptional activity of ERα and induced slow and gradual decrease in the amount of ERα protein (henceforth referred to as down-regulation of ERα). The 4,4′-DHS-induced down-regulation of ERα in MCF-7 cells involved a mechanism that was insensitive to the two most specific proteasome inhibitors, *clasto*-lactacystin-β-lactone and epoxomycin, but sensitive to MG132 at concentrations exceeding that required for maximal inhibition of the proteasome in MCF-7 cells. Therefore, 4,4′-DHS appears to induce down-regulation of ERα by a proteasome-independent mechanism. Here, we present data to show that both 4-OH and 4′-OH are critical for the ability of 4,4′-DHS to induce down-regulation of ERα and suggest that 4,4′-DHS provides a useful scaffold for development of novel ERα antagonists.

1. Introduction

Estrogens are essential for the development and function of numerous tissues, most notably the brain, bone, the cardiovascular system and female reproductive tissues such as the breast and uterus [1]. At the molecular level, the effect of estrogens on cells is primarily mediated by ERα and the β-type estrogen receptor (ERβ) in the nucleus. ERα and ERβ share

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six homologous regions and function as estrogen-dependent transcription factors [2,3]. Binding of estrogen to the ligand-binding domain (LBD) of the ERs causes conformational changes in and activation of the receptors, and formation of receptor homo-dimers, which bind to the *cis* estrogen-responsive elements in the promoters of estrogen-responsive genes [2,3], and stimulate the expression of these genes in the presence of co-activators [4,5]. The estrogen-responsive genes encode the proteins that regulate various estrogen-dependent cellular activities, and hence, regulation of the transcriptional activity and the amount of the ERs is fundamental to the control of the estrogen-dependent activities in cells.

The eukaryotic proteasome is a giant 26S ATP-dependent proteolytic complex, which possesses chymotrypsin-like, trypsin-like and peptidyl glutamyl peptide hydrolase (PGPH) activities that play a major role in the degradation of all short-lived and many long-lived nuclear and cytosolic proteins [6,7]. Therefore, the proteasome is directly or indirectly involved in the regulatory processes of a myriad of cellular activities, including signal-transduction, cell cycle progression, cell proliferation, differentiation and apoptosis [6,7]. Prior to being degraded by the proteasome, a target protein of the proteasome is ubiquitinated, that is, it is covalently modified at a specific lysine residue by a polymer chain of ubiquitin. Ubiquitination of the protein essentially marks the protein for the proteasome to recognize and degrade it [6,7].

Therefore, accumulation of ubiquitinated proteins and up-regulation of various target proteins of the proteasome in cells are indications of reduced activity of the proteasome in the cells.

It has been demonstrated that ERα can be ubiquitinated and degraded by the proteasome in vitro [8], and treatment of various types of cells with the natural estrogen, 17-β-estradiol (E_2), rapidly induces down-regulation of ERα by mechanisms that are sensitive to the proteasome inhibitors, MG132 and *clasto*-lactacystin-β-lactone [8–15]. Furthermore, E_2 induces down-regulation of the wild-type ERα, but not a mutant ERα that lacks ligand-binding activity [11]. Therefore, degradation of the E_2-occupied ERα by the proteasome is responsible for down-regulation of ERα in cells treated with E_2. Also, it has been demonstrated that treatment of cells with various pure ERα antagonists, including ICI-182-780 and GW5638, rapidly induces down-regulation of ERα by mechanisms that are sensitive to MG132 and *clasto*-lactacystin-β-lactone [11–13,16], and that binding of the antagonists to ERα is a prerequisite for these antagonists to induce down-regulation of ERα [11]. Therefore, binding of either an agonist or a pure ERα antagonist to ERα marks it for ubiquitination and subsequent degradation by the proteasome. Currently, most aspects of the proteasome-dependent mechanisms involved in down-regulation of ERα occupied by ligands remain virtually uncharacterized except that the mechanism that down-regulates the E_2-occupied ERα requires physical interaction between ERα and the transcriptional co-activator AIB1, whereas the mechanism, which down-regulates the ICI-182-780-occupied ERα, does not require AIB1 [16]. Unlike the estrogens and pure ERα antagonists, the mixed ERα agonist/antagonist, tamoxifen, induces up-regulation of ERα in cells [11–13,16]. Although the resistance of the tamoxifen-occupied ERα remains mechanistically unclear, it has been suggested that tamoxifen-induced up-regulation of ERα is due to resistance of the tamoxifen-occupied ERα to the proteasome [11–13,16].

Previously, we synthesized numerous hydroxy-*trans*-stilbenes, including 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), and studied their antioxidant and apoptotic activities [17–20]. Interestingly, 4,4′-DHS has been identified by other investigators as a specific ER ligand upon searching for natural and synthetic compounds that can bind to ER [21]. These investigators arbitrarily set the binding affinity of E_2 to ER of rat uterus at 100, and then calculated the relative binding affinities (RBA) of other compounds to the ER using data produced by in vitro competitive-binding assays [21]. It was determined that 4,4′-DHS was a specific ER ligand with an RBA of 0.281, which was much higher than the RBAs of bisphenol (RBA = 0.086) and Phenol Red (RBA = 0.001), two well-known ER agonists [21]. This information prompted us to investigate the effect of 4,4′-DHS on ERα in breast cancer cells, and found that 4,4′-DHS acted as an ERα antagonist and induced down-regulation of ERα.

2. Materials and methods

2.1. Materials

Tamoxifen, Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), leupeptin, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride (PMSF), 17-β-estradiol (E_2), cycloheximide (CHX) and the mouse monoclonal antibodies against β-actin (A-5316), γ-tubulin (GTU-88) and PCNA (PC-10) were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA); MG132 *clasto*-lactacystin-β-lactone, epoxomycin, calpeptin and Z-VAD-FMK were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA); mouse monoclonal antibodies against ERα (F-10), ubiquitin (P4D1) and Hsp90 antibody (F-9) and rabbit polyclonal antibodies against ERα (MC-20), ERβ (H-150), c-Myc (9E10) and hTERT antibody (H-231) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); mouse monoclonal antibodies against topoisomerase 1 (#556597) and cyclin B1 (#554176) from PharMingen (San Diego, CA, USA) and rabbit polyclonal AIB1 antibody (S6505-08) from US Biological (Swampscott, MA, USA). ECL Western blot reagents were obtained from Amersham Pharmacia Biotechnologies Inc. (Piscataway, NJ, USA). The 3x-Vit-ERE-TATA-Luc plasmid, containing a luciferase gene under the control of ER [22] was kindly provided by Dr. Donald P. MacDonnell (Duke University Medical School, Durham, NC, USA). The compounds, 4-hydroxy-*trans*-stilbene (4-HS), 2,4-dihydroxy-*trans*-stilbene (2,4-DHS), 3,4-dihydroxy-*trans*-stilbene (3,4-DHS), 3,5-dihydroxy-*trans*-stilbene (3,5-DHS), 4,4′-dihydroxy-*trans*-stilbene (4,4′-DHS), 3,4,4′-trihydroxy-*trans*-stilbene (3,4,4′-THS) and 4,4′-dimethoxy-*trans*-stilbene (4,4′-DMS) were synthesized as described [17,18], purified by column chromatography and re-crystallized. Mass spectroscopy (on a Bruker APEX II FT-MS spectrometer) and ^{1}H NMR (on a Bruker AM 400 NMR spectrometer) analyses confirmed the structures of the compounds. The purities of the compounds were determined to be 99.5, 99, 99.7, 98.2, 98.3, 99.2 and 99.8% for 4,4′-DHS, 2,4-DHS, 4-HS, 3,4,4′-THS,