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Down-regulation of estrogen receptor-α in MCF-7 human breast cancer cells after proteasome inhibition

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ABSTRACT

The eukaryotic proteasome is a 26S ATP-dependent proteolytic complex, which possesses chymotrypsin-like, trypsin-like and peptidyl glutamyl peptide hydrolase (PGPH) activities, which enable the proteasome to degrade all short-lived and many long-lived proteins, and consequently regulate a myriad of activities in cells. In this study, we observed that inhibition of the proteasome, and more specifically, inhibition of the chymotrypsin-like activity of the proteasome, in MCF-7 human breast cancer cells resulted in selective down-regulation of the nuclear estrogen receptor-α (ERα). Our data indicated that estrogen had no effect, whereas the ERα antagonist, tamoxifen, reduced the amount of ERα that could be subjected to down-regulation after proteasome inhibition. Furthermore, our data demonstrated that protein synthesis was required for the down-regulation of ERα to occur. Collectively, these data indicate the existence of a proteasome-dependent mechanism that is utilized by MCF-7 cells to maintain a steady-state level of ERα.

1. Introduction

Estrogen is an essential hormone of the body and regulates the development and functions of numerous tissues, most notably the brain, bone, the cardiovascular system, and female reproductive tissues such as the uterus and breast [1–4]. At the molecular level, the effect of estrogen on cells is primarily mediated by nuclear receptors, ERα and ERβ, which share six homologous regions and function as transcription factors [5–7]. Binding of estrogen to the ligand-binding domain 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 (EREs) in the promoters of a vast number of estrogen-responsive genes, and stimulate the expression of these genes with the help of co-activators [5,8,9]. The estrogen-responsive genes encode proteins whose functions are essential for the estrogen-dependent cellular functions. When there is estrogen imbalance (over production or inadequate production), dysfunction of estrogen receptors or defects in the pathways of estrogenic signal transduction, developmental and health problems occur in the body. For example, it has been established that long-term estrogen stimulation of epithelial cells with oncogenic mutations in the milk ducts of the breast can lead to the development of

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estrogen-dependent breast cancer [10–12]. Therefore, elucidation of the mechanisms that mediate the effects of estrogen on cells is of great importance to understand how estrogen controls the development of the body and to develop therapies to treat various estrogen-related diseases.

The eukaryotic proteasome is a 26S ATP-dependent proteolytic complex, which consists of multiple copies of 32 different proteins that form a cylindrical 20S core and two 19S regulatory caps that sandwich the 20S core. The 20S core possesses chymotrypsin-like, trypsin-like and peptidyl glutamyl peptide hydrolase (PGPH) activities, which degrade all short-lived and many long-lived proteins in the nucleus and cytosol [13,14]. Therefore, the proteasome ultimately regulates a myriad of cellular processes, including signal-transduction, cell cycle progression, cell proliferation and differentiation, and apoptosis [13,14]. A protein to be degraded by the proteasome is covalently modified with a polymer chain of ubiquitin (Ub) by a series of reactions. Three enzymes, namely El (Ub-activating enzyme), E2 (Ub-conjugating enzyme), and E3 (Ub-ligase), are responsible for adding ubiquitin molecules to the proteasome target proteins [13,14]. The 19S cap of the proteasome recognizes and unfolds the ubiquitinated protein, and then delivers it through the opening and into the cavity of the 20S core, where the protein is cleaved into small peptide pieces by the chymotrypsin-like, trypsin-like, and PGPH enzymes [13,14]. Therefore, one of the earliest hallmarks of proteasome inhibition in cells is the accumulation of a large quantity of ubiquitinated proteins and up-regulation of the target proteins of the proteasome in various cellular compartments.

Because of the complexity of the proteasome, molecular approaches targeting individual components in the proteasome have in general proven unsuccessful in determining proteasome-dependent activities in mammalian cells. However, several cell membrane permeable proteasome inhibitors, most notably MG132, *clasto*-lactacystin β-lactone and epoxomycin, have been used to inhibit the proteasome and consequently inhibit proteasome-dependent activities in mammalian cells [15–17]. At the biochemical level, MG132 [18], *clasto*-lactacystin β-lactone [19,20] and epoxomycin [21,22] all inhibit both the chymotrypsin-like and trypsin-like activities of the proteasome, whereas the two newly developed inhibitors, YU101 [23] and YU102 [23,24], selectively inhibit the chymotrypsin-like activity and PGPH activity of the proteasome, respectively. Currently, the use of these inhibitors is indispensable for the identification of proteasome-dependent activities in cells.

Recently, we initiated an investigation of proteasome-dependent activities in the MCF-7 human breast cancer cell line, which has been the most widely used human breast cancer cell line in studies of estrogen-dependent activities in breast cancer. During the course of this investigation, we observed that treatment of MCF-7 cells with the proteasome inhibitor, MG132, but not with the inhibitors of several classes of non-proteasomal proteases, resulted in selective down-regulation of ERα. This observation suggested that the proteasome was required to maintain an adequate amount of ERα in MCF-7 cells, and prompted us to conduct additional experiments to assess the relationship between proteasome inhibition and ERα down-regulation in MCF-7 cells.

2. Materials and methods

2.1. Materials

Na-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), leupeptin, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride, 3,4-dichloroisocoumarin (DICI), 17-β-estradiol (E_2), and β-actin monoclonal antibody (A-5316) were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA); MG132, *clasto*-lactacystin β-lactone, epoxomycin, YU101, calpeptin, and Z-VAD-FMK were from Calbiochem–Novabiochem Corp. (La Jolla, CA, USA); and YU102 was purchased from BIOMOL (Plymouth Meeting, PA). Mouse monoclonal antibodies against human ERα (F-10), Hsp-90 (F-9), IκB-α (H-4), PARP (F-2), ubiquitin (P4D1), and polyclonal rabbit antibodies against human p21 (C-19), Daxx (M-112), c-Myc (9E10), STAT1 (C-20), STAT3 (H-190), and TERT (H-231) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and mouse monoclonal antibodies against human cyclin B1 (554176), DNA polymerase δ (DNA pol δ) (D73020), Rb (14001A) and topoisomerase I (Top1) (#556597) were purchased from PharMingen (San Diego, CA, USA). ECL Western blot reagents were obtained from Amersham Pharmacia Biotechnologies Inc. (Piscataway, NJ, USA).

2.2. Cell cultures

The MCF-7 cell line was originally obtained from American Type Culture Collection (Manassas, VA), and cultured in DMEM supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (50 units/ml). All cell cultures were maintained in a 37 °C humidified incubator containing 5% CO_2-atmosphere. To deprive MCF-7 cells of estrogen, the MCF-7 cell cultures at 50% confluence were washed twice with serum- and phenol red-free medium, and then incubated in phenol red-free medium supplemented with 2% of charcoal-stripped fetal bovine serum for 96 h with a medium change every 24 h. Then, the cells were treated with various agents.

2.3. Treatment of cells and Western blot analysis

Nearly confluent cell cultures were gently trypsinized, and the detached cells were subsequently washed and suspended in fresh culture medium, plated into culture dishes at approximately 50% confluence, and cultured for 48 h. Then, the cells were left untreated or treated with various agents for 24 h. After the treatment, the cells were scraped off the dish with a latex rubber cell-remover, and pelleted in a tube by centrifugation. The cell pellet was washed twice in PBS, and then subjected to whole cell protein extraction. Protein concentrations in the extracts were determined using the BIO-RAD Protein Assay reagents. Aliquots (50 μg/sample) of whole cell extracts were subjected to SDS-PAGE and Western blot analyses to detect various proteins using ECL reagents [25]. The intensity of the protein bands on a Western blot was estimated using the NIH 1.6.3 software.

2.4. Cell fractionation

MCF-7 cells were removed from the culture dishes, washed in PBS, and lysed in ice-cold CSK buffer [10 mM Pipes, 300 mM

sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 2× protease inhibitor cocktail (Roche, Cat# 1783580), 0.5% Triton X-100, pH 6.8] according to a published protocol [26]. The lysate was centrifuged at 12,500 × *g* for 5 min at 4 °C, and the supernatant (cytosolic or C-fraction) and pelleted materials (nuclear or N-fraction) were saved. The C- and N-fractions were adjusted to equal final volume of IX sample buffer (100 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 4 M urea, 5% β-mercaptoethanol, 0.06% bromophenol blue), heated for 5 min at 100 °C, and aliquots were analyzed by the Western blotting technique for the presence of specific proteins.

3. Results

3.1. *Inhibition of the proteasome in MCF-7 cells results in down-regulation of ERα*

The proteasome inhibitor, MG132 [18], has been used in virtually all investigations of the effect of proteasome inhibition on various activities and functions of cultured mammalian cells. Therefore, we first investigated the effectiveness of MG132-mediated inhibition of the proteasome in MCF-7 cells. In this investigation, we treated MCF-7 cells for 24 h with 0.5–5 μM of MG132, and then determined the extent of MG132-mediated inhibition of the proteasome in the cells by the amount of MG132-induced intracellular accumulation of ubiquitinated proteins, a hallmark of proteasome inhibition in cells. The results from Western blot analysis of the proteins in MCF-7 cells, untreated and treated with MG132, showed that MG132 induced intracellular accumulation of a vast amount of ubiquitinated proteins in a dose-dependent fashion (Fig. 1A), indicating dose-dependent inhibition of the proteasome by MG132. Furthermore, the results showed that the 24-h treatment of MCF-7 cells with approximately 1.5 μM of MG132 induced maximal accumulation of ubiquitinated proteins, i.e. maximal inhibition of the proteasome in the cells (Fig. 1B). Effective inhibition of the proteasome in MCF-7 cells by the 24-h treatment with 1.5 μM of MG132 was demonstrated by up-regulation of the well-characterized proteasome target proteins, cyclin B1, cyclin D3, IkBα, and c-Myc [27–30], in the cells (Fig. 2B). Subsequently, we investigated the effect of treating MCF-7 cells with MG132 on the steady-state levels of a large group of proteins that control a wide range of cellular factions, including ERα that controls estrogen responses in MCF-7 cells. The results showed that treatment of MCF-7 cells for 24 h with 1.5 μM of MG132 induced almost complete down-regulation of ERα (Fig. 2), but virtually had no effect on the levels of many other proteins (Fig. 2B). Therefore, it appeared that inhibition of the proteasome caused very selective down-regulation of ERα in MCF-7 cells.

To investigate if inhibition of other proteases in MCF-7 cells would induce down-regulation of ERα, we treated MCF-7 cells for 24 h with calpeptin (a relatively specific calpain inhibitor), 3,4-dichloroisocoumarin (a general inhibitor of serine proteases), Z-VAD-FMK (a pan-caspase inhibitor), leupeptin (an inhibitor of cysteine and serine proteases), TLCK (a general inhibitor of trypsin-like serine proteases), and TPCK (a general inhibitor of chymotrypsin-like serine proteases), and then determined the effect of these inhibitors on the proteasome and the level of ERα. The results showed that none of these inhibitors induced either

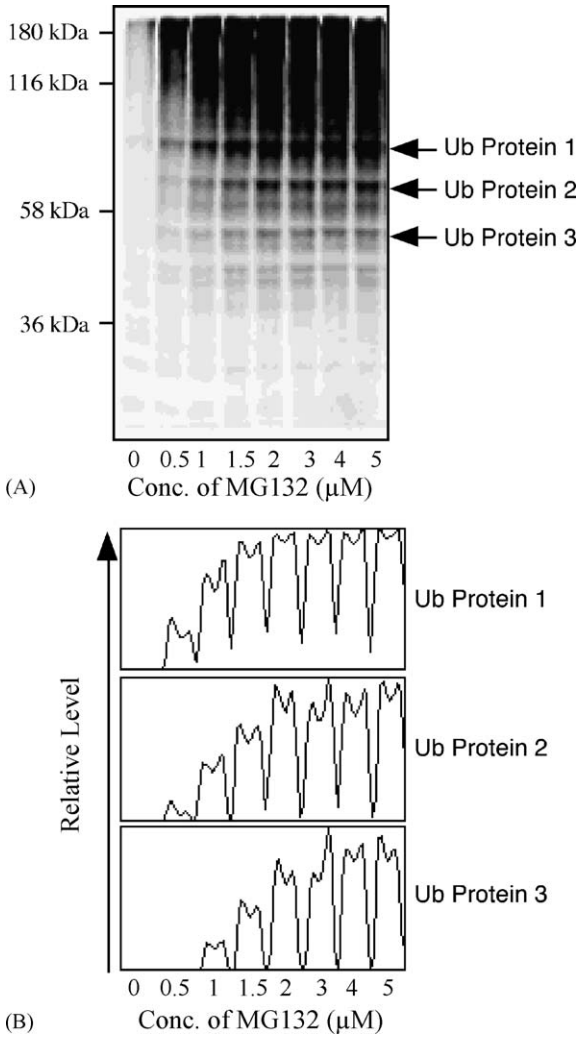

Fig. 1 – MG132 induces proteasome inhibition in MCF-7 cells. (A) Western blotting detection of the ubiquitinated proteins (Ub proteins) in MCF-7 cells treated for 24 h with various concentrations of MG132. The amount of the accumulated Ub proteins is proportional to the extent of the MG132-mediated inhibition of the proteasome. (B) The intensities of the three clearly defined bands of Ub proteins in the Western blot of (A) were estimated with the aid of the NIH 1.6.3 imaging software.

accumulation of ubiquitinated proteins (i.e. inhibition of the proteasome) or down-regulation of ERα (Fig. 3). Collectively, the results in Figs. 2 and 3 strongly suggested the existence of a specific relationship between proteasome inhibition and the down-regulation of ERα in MCF-7 cells.

To confirm the relationship between proteasome inhibition and down-regulation of ERα, we treated MCF-7 cells for 24 h with various concentrations of the proteasome inhibitors, *clasto*-lactacystin β-lactone and epoxomycin [19–22], and then assessed their effect on the activity of the proteasome and the level of ERα in MCF-7 cells. The results showed that both *clasto*-lactacystin β-lactone (Fig. 4A) and epoxomycin (Fig. 4B) induced, in a dose-dependent fashion, accumulation of ubiquitinated proteins (i.e. inhibition of the proteasome) and down-regulation

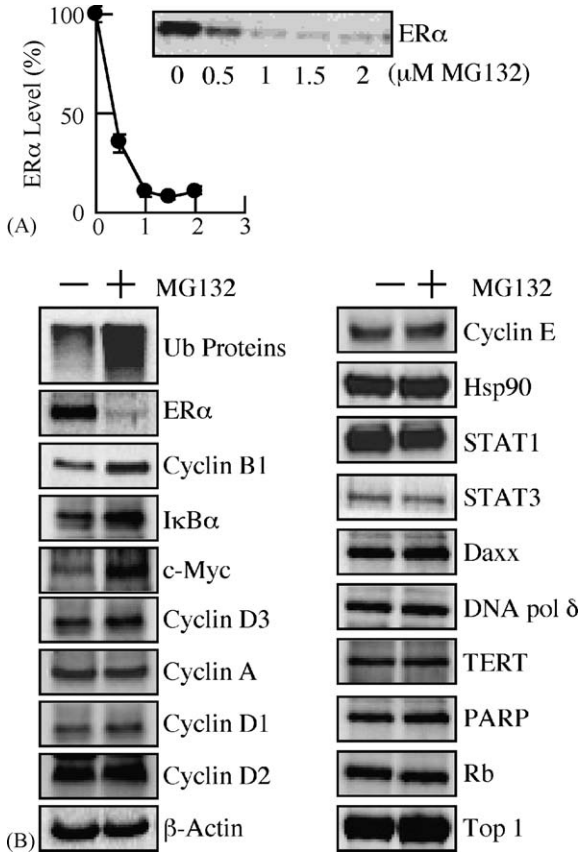

Fig. 2 – MG132 induces selective down-regulation of ERα. (A) MCF-7 cells were treated for 24 h with 0, 0.5, 1, 1.5, and 2 μM of MG132, and then subjected to Western blotting analysis to detect ERα. The amount of ERα in the Western blot was quantitatively estimated with the aid of the NIH 1.6.3 imaging software. (B). MCF-7 cells were untreated and treated for 24 h with 1.5 μM of MG132, and then subjected to Western blotting analysis to detect the listed proteins.

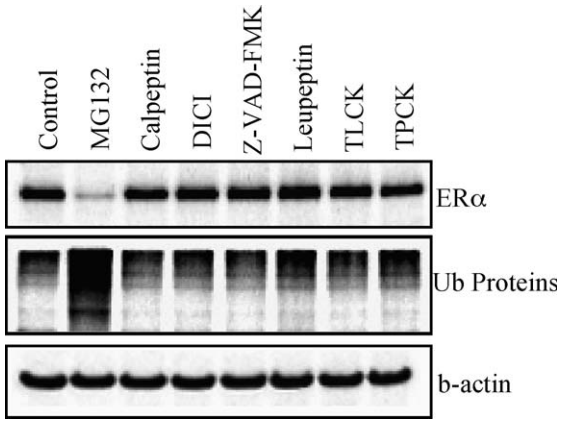

Fig. 3 – Inhibition of the proteasome, but not other proteases, induces ERα down-regulation. MCF-7 cells were untreated or treated for 24 h with 1.5 μM of MG132, 100 μM of calpeptin, 50 μM of DICI (3,4-dichloroisocoumarin), 100 μM Z-VAD-FMK, 50 μM leupeptin, 50 μM TLCK, and 50 μM TPCK. Then, whole cell extracts were subjected to Western blotting analysis to detect ERα and ubiquitinated proteins (Ub proteins).

of the ERα. These results indicated that proteasome inhibition was indeed responsible for the occurrence of ERα down-regulation. To further investigate whether inhibition of a particular activity or all three proteolytic activities were required to induce down-regulation of ERα, we investigated the effect of the compounds, YU101 and YU102, which specifically inhibit the chymotrypsin-like activity and the PGPH activity of the proteasome, respectively [23,24]. The results showed that treatment of MCF-7 cells for 24 h with YU101 (Fig. 4C), but not YU102 (Fig. 4D), induced significant accumulation of ubiquitinated proteins and down-regulation of ERα in a dose-dependent fashion. This finding indicated that inhibition of the chymotrypsin-like activity of the proteasome alone was sufficient to cause down-regulation of ERα in MCF-7 cells.

3.2. *Down-regulation of ERα occurs in the nucleus after substantial inhibition of the proteasome*

To investigate (i) whether down-regulation of ERα occurs in the nucleus or the cytoplasm after proteasome inhibition and (ii) the relationship between the extent of proteasome inhibition and the degree of ERα down-regulation, we treated MCF-7 cells with 1.5 μM of MG132 for various lengths of time (h), and then processed the cells to yield cytosolic and nuclear fractions, which were subsequently analyzed for presence of ERα and ubiquitinated proteins. The results showed time-dependent accumulation of ubiquitinated proteins (i.e. time-dependent inhibition of the proteasome) in both the cytoplasmic and nuclear fractions, specific nuclear localization of ERα, and time-dependent down-regulation of ERα in the nucleus (Fig. 5A). A maximal accumulation of the ubiquitinated proteins (i.e. maximal proteasome inhibition) occurred after the cells had been treated for 14–16 h (Fig. 5B), whereas a significant down-regulation of ERα occurred after the cells were treated for 16 h (Fig. 5B). These data demonstrated that down-regulation of ERα was a nuclear event, which occurred after substantial inhibition of the proteasome.

3.3. *Differential effects of estrogen and tamoxifen on the amount of ERα that could be down-regulated after proteasome inhibition*

The MCF-7 cells used in the above experiments were cultured in medium containing the estrogenic dye, phenol red, and 10% fetal bovine serum, which can contribute up to 10 pg/ml of 17-β-estradiol (E_2). To investigate whether ER ligands would affect the amount of ERα that could be subjected to down-regulation after proteasome inhibition, we first deprived MCF-7 cells of estrogen and then treated the cells for 24 h with 1.5 μM of MG132 in the absence and presence of a high concentration of E_2 or the ERα antagonist tamoxifen. Then we determined the effect of MG132, in the absence and presence of E_2 or tamoxifen, on the amount of ubiquitinated proteins and ERα in the cells. The results showed that: (i) both E_2 and tamoxifen had no effect on the ability of MG132 to inhibit the

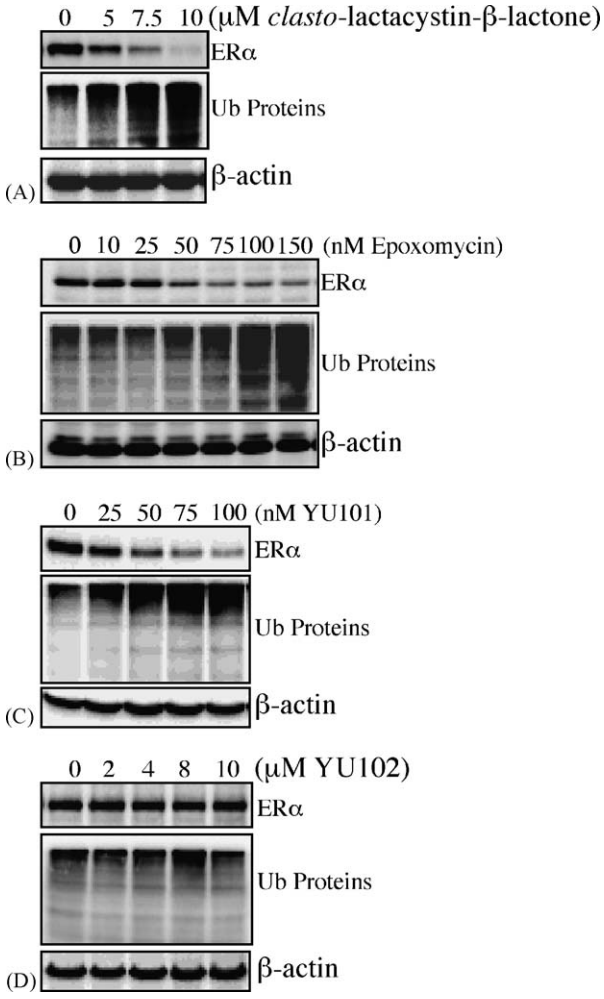

Fig. 4 – Proteasome inhibitors induce down-regulation of ERα. Western blotting detection of the ubiquitinated proteins (Ub proteins) and ERα in MCF-7 cells untreated and treated with various concentrations of (A) *clasto*-lactacystin β-lactone, (B) epoxomycin, (C) YU101, and (D) YU102.

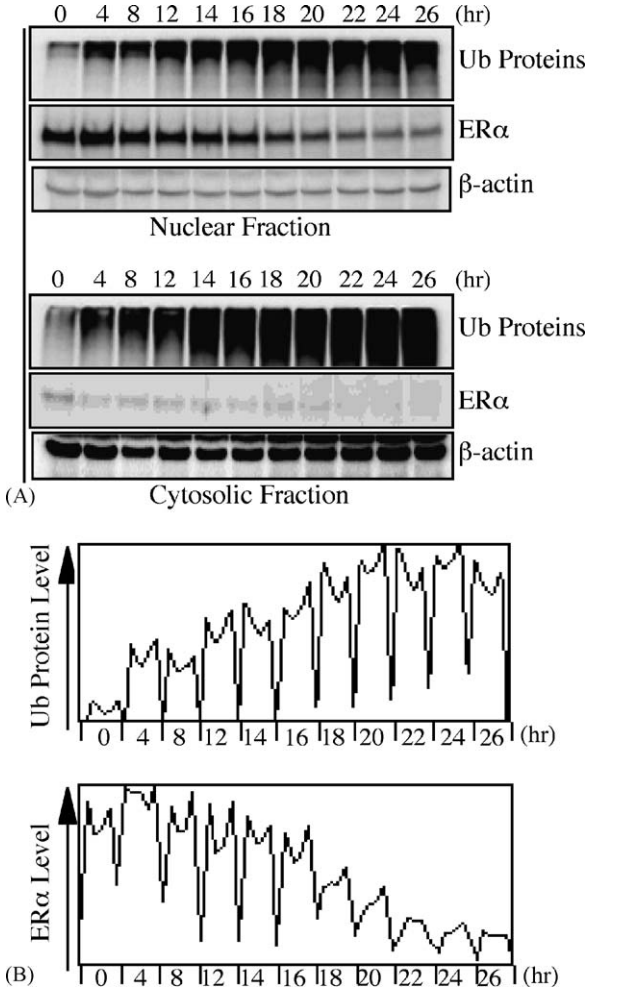

Fig. 5 – Down-regulation of ERα occurs in the nucleus after proteasome inhibition. MCF-7 cells were treated with 1.5 μM of MG132 for various periods of time (h), and then were processed to yield nuclear and cytosolic fractions, which were subjected to Western blot analysis to detect ubiquitinated proteins (Ub proteins) and ERα. The intensities of the ERα and Ub proteins in the nuclear fractions were estimated with the aid of the NIH 1.6.3 imaging software.

proteasome and consequently induce accumulation of ubiquitinated proteins (Fig. 6); (ii) E_2 had no effect on the amount of ERα that was subjected to down-regulation (Fig. 6); (iii) treatment of MCF-7 cells with tamoxifen alone induced up-regulation (by approximately 86%) of ERα (Fig. 6B), which is a characteristic effect of tamoxifen on ERα in cells [31–34]; (iv) tamoxifen significantly reduced the amount of ERα that could be down-regulated in the presence of MG132 (Fig. 6). Therefore, E_2 and tamoxifen had differential effects on the amount of ERα that could be reduced after proteasome inhibition.

3.4. *Cycloheximide blocks the ability of MG132 to induce down-regulation of ERα*

To investigate whether inhibition of the synthesis of ERα for 24 h in MCF-7 cells would result in complete down-regulation of ERα, we cultured MCF-7 cells for 24 h in the absence and presence of 20 μM of the translation inhibitor, CHX, and then determined the effect of CHX on the level of ERα in the cells. The results showed that the CHX treatment reduced the cellular level of ERα by approximately 50% (Fig. 7), indicating that the natural ERα turnover rate was relatively slow, and hence, inhibition of ERα synthesis for 24 h alone could not cause complete down-regulation of ERα in MCF-7 cells. Next, we investigated whether down-regulation of ERα after proteasome inhibition would occur faster in the presence of CHX. In the investigation, we treated MCF-7 cells for 24 h with 1.5 μM of MG132 in the presence of 20 μM of CHX and then determined the effect of MG132 on the level of ERα. The result demonstrated that the amount of ERα in the cells treated with MG132 + CHX was almost the same as that in the cells treated with CHX (Fig. 7). This finding indicated that MG132 no longer induced down-regulation of ERα in the presence of CHX. Therefore, synthesis of a new gene

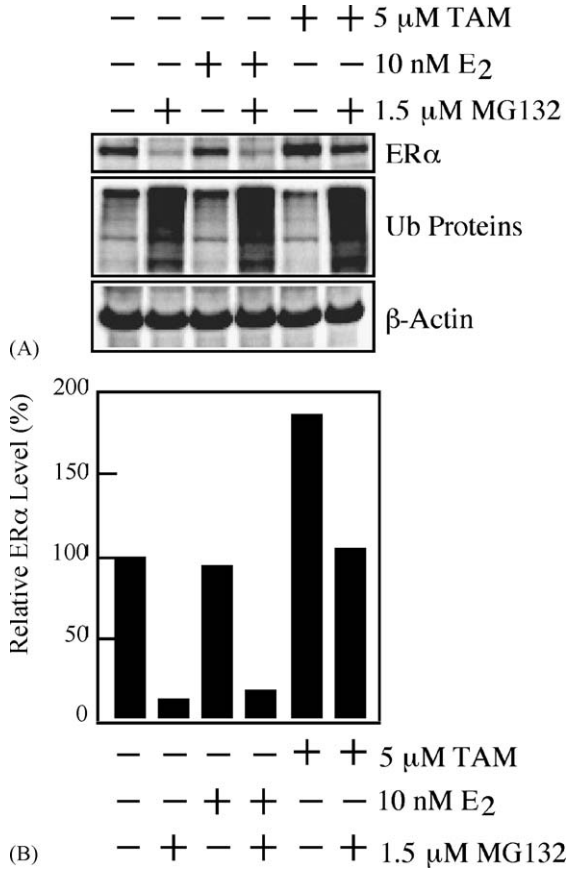

Fig. 6 – E_2 and tamoxifen have differential effects on the amount of down-regulated ERα after proteasome inhibition. (A) Western blotting detection of the ubiquitinated proteins (Ub proteins) and ERα in estrogen-deprived MCF-7 cells after they were untreated or treated for 24 h with 1.5 μM of MG132 in the absence or presence of 10 nM E_2 or 5 μM tamoxifen (TAM). (B) The intensities of the ERα protein bands in the Western blot (A) were estimated with the aid of the NIH 1.6.3 imaging software.

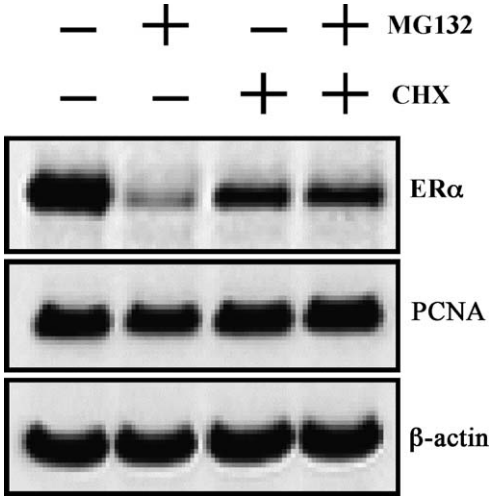

Fig. 7 – CHX blocks the ability of MG132 to induce down-regulation of ERα. Western blotting detection of ERα and the control proteins, PCNA and β-actin, in MCF-7 cells untreated or treated for 24 h with 20 μM of CHX in the absence and presence of 1.5 of MG132.

product(s) was required for the down-regulation of ERα to occur after proteasome inhibition in MCF-7 cells.

4. Discussion

The proteasome inhibitors, most notably MG132 and *clasto*-lactacystin β-lactone, have been widely used to treat cells for the purpose of inhibiting proteasome-dependent mechanisms [15–17]. In this study, we observed that treatment of MCF-7 cells with the proteasome inhibitors, MG132, *clasto*-lactacystin β-lactone and epoxomycin (Figs. 2 and 4), but not inhibitors of several other classes of proteases (Fig. 3), inhibited the activity of the proteasome and concomitantly induced selective down-regulation of ERα in the cells. Furthermore, we observed that treatment of MCF-7 cells with YU101, which is a selective inhibitor of the chymotrypsin-like activity of the proteasome [23], but not YU102, which selectively inhibits the PGPH activity of the proteasome [23,24], induced down-regulation of ERα in the cells (Fig. 4). Since MG132, *clasto*-lactacystin β-lactone and epoxomycin all primarily inhibit the chymotrypsin-like activity of the proteasome [16,17], our observations collectively indicate that inhibition of the chymotrypsin-like activity of the proteasome resulted in the down-regulation of ERα in MCF-7 cells.

Since CHX-mediated inhibition of protein synthesis for 24 h reduced the level of ERα by approximately 50% (Fig. 7), and proteasome inhibition for 24 h resulted in down-regulation of a much higher amount of ERα in MCF-7 cells (Figs. 2 and 4), we concluded that down-regulation of ERα after proteasome inhibition was not the result of passive ERα turnover at a normal pace, but was due to active degradation of the ERα. Furthermore, failure of inhibition of the proteasome in the presence of CHX to reduce the cellular level of ERα (Fig. 7) strongly suggested that synthesis of a protein(s) was required for down-regulation of ERα to occur after proteasome inhibition. Therefore, we propose that MCF-7 cells utilize the chymotrypsin-like activity of the proteasome to suppress a mechanism that actively degrades ERα. It is very likely that inhibition of the chymotrypsin-like activity of the proteasome in MCF-7 cells results in the expression of a protease, which specifically degrades ERα. Thus, CHX-mediated inhibition of protein synthesis in MCF-7 cells blocked the expression of this putative ERα protease, and consequently prevented down-regulation of ERα after proteasome inhibition.

The fact that down-regulation of ERα after proteasome inhibition was not affected by E_2, but was significantly blocked by tamoxifen (Fig. 6), suggests that this putative ERα protease degrades ERα that is either un-occupied or occupied by E_2, but not ERα occupied by tamoxifen. It is also likely that inhibition of the chymotrypsin-like activity of the proteasome in MCF-7 cells results in the expression of a factor(s), which interacts with ERα that is unoccupied or occupied by E_2, and marks the ERα for degradation by a pre-existing protease; furthermore, this putative factor(s) is unlikely to interact with ERα occupied by tamoxifen.

Nevertheless, it is plausible that the tamoxifen-occupied ERα assumes a conformation that is not recognized by the factor(s) that induces down-regulation of ERα.

In summary, our results demonstrate that MCF-7 cells utilize the chymotrypsin-like activity of the proteasome in a mechanism that maintains a steady-state level of ERα.

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