

Implication of proteasome in estrogen receptor degradation

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Abstract In MCF-7 breast cancer cells, estradiol (E_2) and pure antiestrogen RU 58668 down-regulate the estrogen receptor (ER). Interestingly, the protein synthesis inhibitor cycloheximide (CHX) abrogated solely the effect of E_2 suggesting a selective difference in the degradation of the receptor induced by estrogenic and antiestrogenic stimulations. A panel of lysosome inhibitors (i.e. bafilomycin, chloroquine, NH_4Cl , and monensin), calpain inhibitors (calpastatin and PD 150606) and proteasome inhibitors (lactacystin and proteasome inhibitor I) were tested to assess this hypothesis. Among all inhibitors tested, lactacystin and proteasome inhibitor I were the sole inhibitors to abrogate the elimination of the receptor induced by both E_2 and RU 58668; this selective effect was also recorded in cells prelabeled with [3H]tamoxifen aziridine before exposure to these ligands. Hence, differential sensitivity to CHX seems to be linked to the different mechanisms which target proteins for proteasome-mediated destruction. Moreover, the two tested proteasome inhibitors produced a slight increase of ER concentration in cells not exposed to any ligand, suggesting also the involvement of proteasome in receptor turnover.

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Key words: Proteasome; Lysosome; Calpain; Estrogen receptor; MCF-7 cell

1. Introduction

There is a continuous turnover of proteins in eukaryotic cells, with individual half-life varying from a few minutes to weeks [1]. Among proteins with the shortest half-lives are key regulatory factors. Metabolic instability of these factors allows a rapid adjustment of their concentrations and/or subunit composition through changes in their rate of synthesis or degradation.

The estrogen receptor (ER) is a transcriptional factor which regulates cellular genes upon ligand binding [2,3]. While ER activation and function have been extensively studied, knowledge of the mechanisms governing its concentration is rather weak. The half-life of ER is about 3 to 4 h, as revealed by pulse-chase experiments employing covalently attached ligands or dense amino acid incorporation-density shift techniques [4–6]. Analysis of the underlying degradation process is of importance since it governs the receptor level and, consequently, influences the response of the cells to estrogens as well as their antagonists [7,8].

Mammalian cells contain two distinct proteolytic pathways: lysosomal and non-lysosomal [9]. The ability of each system

to partly or completely degrade proteins, reflects the complexity of intracellular proteolysis [10,11]. With regard to the lysosomal pathway, several mechanisms for the uptake of cellular proteins into lysosomes have been described including the recognition of specific amino acid sequences [12]. Of note, among non-lysosomal systems, calpains which are calcium-dependent cysteine proteases, were reported to hydrolyse ER extracted from uterine and breast cancer tissues [13,14]. There are at least two types of calpains, μ - and m -calpains (also designed as calpain I and calpain II), requiring respectively a micromolar and a millimolar concentration of Ca^{2+} for activation [15]. In addition to calpains, cells contain another major non-lysosomal proteinase system known as the 26S proteasome complex [16–18]. Although the latter is predominantly a cytosolic system, it has also been detected in the nucleus, and purified from erythrocyte membranes [19,20]. The '20S catalytic core' of the proteasome contains several subunits (α - and β -subunits) that allow specialized proteolytic functions. These subunits exhibit up to five different peptidase activities, including sites that preferentially cleave after basic, hydrophobic, or acidic residues [21–23].

In the present study, we tried to elucidate the degree of implication of these pathways in the degradation of ER. Our study was conducted on MCF-7 breast cancer cells which have been widely used to study the regulation of ER level under both estrogenic and antiestrogenic stimulations. In these cells, estrogens (i.e. estradiol, E_2 or diethylstilbestrol) and pure antiestrogens (i.e. RU 58668 or ICI 164384), decrease ER level within a few hours (a down-regulation process also called 'processing' [24,25]). Protein synthesis inhibitors (i.e. cycloheximide 'CHX' and puromycin) abrogate the down-regulating effect of E_2 without interfering with receptor activation and subsequent transcription ability [26]. Interestingly, the inhibitory effect of CHX did not hold for the receptor elimination induced by pure antiestrogens [25]. This observation led us to also analyse whether ER degradation induced by E_2 and RU 58668 is linked to different proteolytic pathways.

2. Materials and methods

2.1. Reagents

[3H] E_2 (95 Ci/mmol) and [3H]tamoxifen aziridine ([3H]TAZ) (20 Ci/mmol) were purchased from Amersham (UK). Unlabeled E_2 , cycloheximide (CHX), puromycin, NH_4Cl , monensin, chloroquine, bafilomycin and anti-rat IgG agarose were obtained from Sigma (St. Louis, MO, USA). Lactacystin, proteasome inhibitor I, calpastatin and calpain inhibitor PD 150606 were purchased from Calbiochem (San Diego, CA, USA). RU 58668 was a gift of Roussel-Uclaf (Romainville, France). H_{222} rat anti-ER monoclonal antibody was provided by Dr. D. Cotter (Abbott laboratories, North Chicago, IL, USA).

2.2. Cell culture

MCF-7 cells were maintained in culture in Falcon T 75 flasks at 37°C in MEM supplemented with 10% heat-inactivated fetal calf se-

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rum (56°C, 1 h), L-glutamine, penicillin, streptomycin and gentamicin at the usual concentration [27]. Experiments were conducted with 10% fetal calf serum stripped of endogenous hormones by a dextran-coated charcoal (DCC) treatment [28].

2.3. Assessment of proteolytic inhibitors on ligand-induced ER elimination

MCF-7 cells from a near-confluent culture were incubated for up to 6 h either with 1 nM E₂ or 10 nM RU 58668 in the presence or absence of bafilomycin at 10 µM, lactacystin at 10 µM or calpastatin at 0.05 µg/ml. At the end of incubation, cells were washed twice with Ca²⁺/Mg²⁺ free Hank's balanced salt solution (HBSS) and harvested by incubation with 1 mM EDTA in the same buffer (10 to 15 min). All subsequent steps were performed at 0–4°C. After centrifugation (800×g, 10 min), the cell pellets were washed successively with Hank's solution and a phosphate buffer (10 mM potassium phosphate pH 7.4, 10% glycerol, 10 mM thioglycerol and 1.5 mM EDTA). Cellular extracts were obtained by freezing these pellets at –70°C and subsequent homogenization in 1 ml of this phosphate buffer containing 500 mM KCl; cellular debris were removed by ultracentrifugation (100 000×g; 1 h). ER contents of these extracts were measured by the Abbott's enzyme immunoassay (ER-EIA); values were always within the linear range of the kit standard (0–500 fmol/ml). Protein contents of the extracts were assessed by the coomassie Protein Reagent Assay (Pierce, Rockford, IL, USA) using bovine serum albumin (Sigma, fraction V) as standard. ER concentration (fmol/mg protein) was expressed as a percentage of the level in untreated cells (control condition).

2.4. Assessment of proteolytic inhibitors on ligand-induced [³H]TAZ-ER complex elimination

MCF-7 cells were incubated for 1 h with 20 nM [³H]TAZ; for non-specific binding assessment, additional cells were preincubated for 30 min with 1 µM unlabeled E₂ and maintained with the hormone during the whole [³H]TAZ labeling period. After removal of the medium, cells were rinsed three times with 10 ml of MEM at 37°C. Fresh medium containing 1 nM E₂ or 10 nM RU 58668 either with or without bafilomycin at 10 µM, lactacystin at 10 µM or calpastatin at 0.05 µg/ml was then added and the culture pursued up to 6 h. Medium was finally removed and the cells harvested, suspended and extracted as described above. Extracts were added with dimethylformamide (final concentration 7%) and submitted to immunoprecipitation with H₂₂₂ anti-ER (1 µl/ml, 2 h at 4°C). Immune complexes were adsorbed on anti-rat IgG agarose (overnight incubation at 4°C), centrifuged and washed three times in 1 ml of 20 mM Tris-HCl, pH 7.4 containing 500 mM KCl, 1 mM DTT, 50 mM NaF, 10 mM Na₂MoO₄. Pellets were resuspended in 200 µl of the same buffer and the radioactivity of a 120 µl aliquot measured by liquid scintillation counting with an efficiency of ~40% (Scintillation fluid: Ecoscint H; National Diagnostic, Atlanta, GA, USA). Specific [³H]TAZ labeling was calculated and the values were expressed as a percentage of the level in control cells.

2.5. Assessment of proteolytic inhibitors on [³H]E₂ binding capacity

MCF-7 cells were incubated with 1 nM [³H]E₂ in the presence of a given inhibitor for up to 6 h (CHX at 50 µM, NH₄Cl at 10 mM, monensin at 10 µM, chloroquine at 100 µM, bafilomycin at 10 µM,

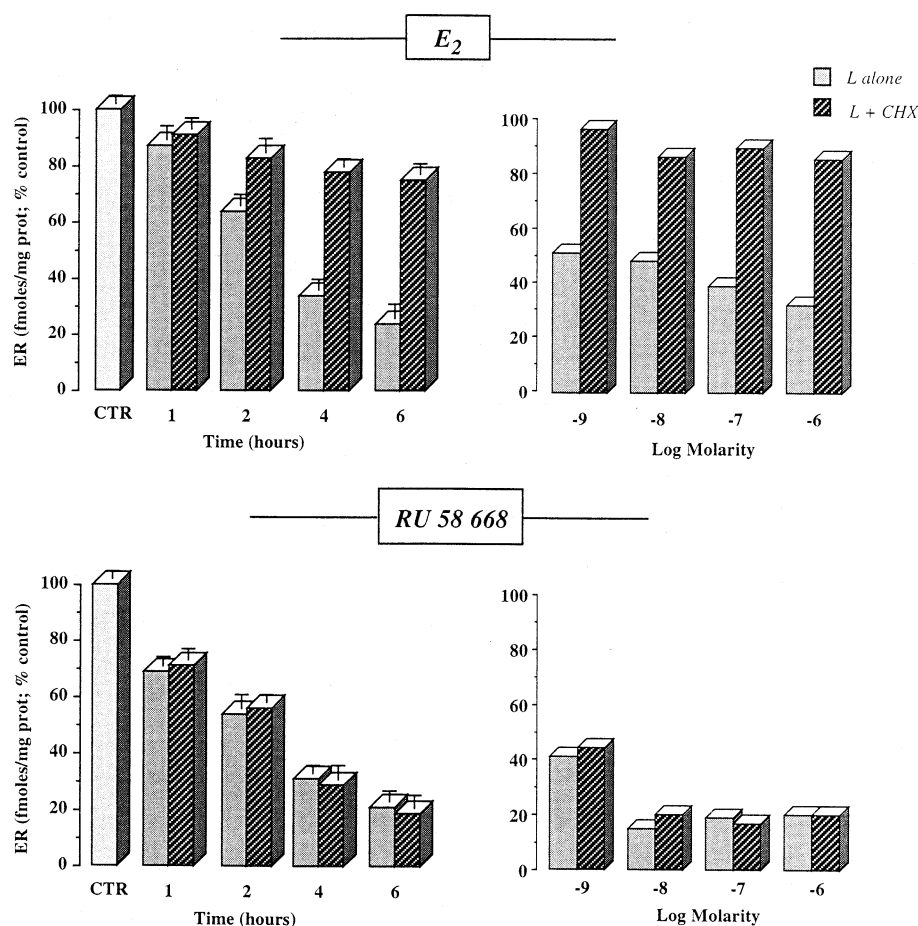


Fig. 1. Effect of CHX on the ligand-induced ER elimination. MCF-7 cells were incubated up to 6 h with 1 nM E₂ or 10 nM RU 58668 in the presence or absence of 50 µM CHX (L: ligand). After removal of the medium, cells were washed, harvested, extracted and their ER contents measured by the Abbott's enzyme immunoassay (ER-EIA). Control cells were incubated without drugs. The data refer to the mean ± S.D. of three separate experiments (CTR: 760, 786, 804 fmol/mg protein = 100%). Additionally, cells were incubated for 3 h with E₂ or RU 58668 at concentrations ranging from 1 nM to 1 µM in the presence or absence of 50 µM CHX; residual ER contents were similarly measured by EIA.

lactacystin at 10 μ M, proteasome inhibitor I at 0.1 nM, calpastatin at 0.05 μ g/ml and PD 150606 at 0.5 μ M). At the given concentration for the duration of our experiment, all these drugs failed to show any cytotoxic effect (MTT assay). Non-specific binding was established by a parallel incubation with a 500-fold excess of unlabeled E_2 . Control [3 H] E_2 labeled cells were maintained without any inhibitor. At the end of the incubation, medium was removed and the cells washed twice with ice-cold saline solution (HBSS). Bound [3 H] E_2 was extracted from the monolayers by an incubation in 250 μ l ethanol at room temperature (20 min) and measured (200 μ l of extract) by scintillation counting. Specifically bound [3 H] E_2 was calculated from the difference of incorporated radioactivity after incubation in the presence and absence of unlabeled E_2 . Data were expressed as percentages of specifically incorporated [3 H] E_2 in the control condition.

In an additional experiment, MCF-7 cells were preincubated up to 6 h with 10 μ M lactacystin or 0.1 nM proteasome inhibitor I before being exposed to [3 H] E_2 ; control cells were maintained without drugs. After removal of the medium, cells were rinsed twice with MEM at 37°C and the culture pursued for 1 h with [3 H] E_2 and one of these two inhibitors. Specific [3 H] E_2 incorporation was subsequently determined as above. At the end of experiment, ER levels were also measured by Abbott's enzyme immunoassay on both control and treated cells (6 h of incubation).

3. Results

3.1. Effect of protein synthesis inhibitor on ligand-induced ER elimination

Treatment of MCF-7 cells with either 1 nM E_2 or 10 nM RU 58668 is known to produce a rapid decrease of their ER level as confirmed here by Abbott's enzyme immunoassays. As expected addition of 50 μ M CHX to the medium at the same time as these ligands abrogated solely the effect of E_2 (Fig. 1, left panel). This property could not be ascribed to a difference of ligand concentration since it occurred for both E_2 and RU 58668 on a wide range of concentrations (Fig. 1, right panel). Interestingly, similar results were observed with 50 μ M puromycin (fmoles/mg protein, % of control: E_2 /Pur = 79, RU 58668/Pur = 18; $n = 2$) clearly establishing the involvement of protein synthesis in the phenomenon. Hence, a major difference in the metabolic degradative pathways following the binding of these ligands to ER should be considered.

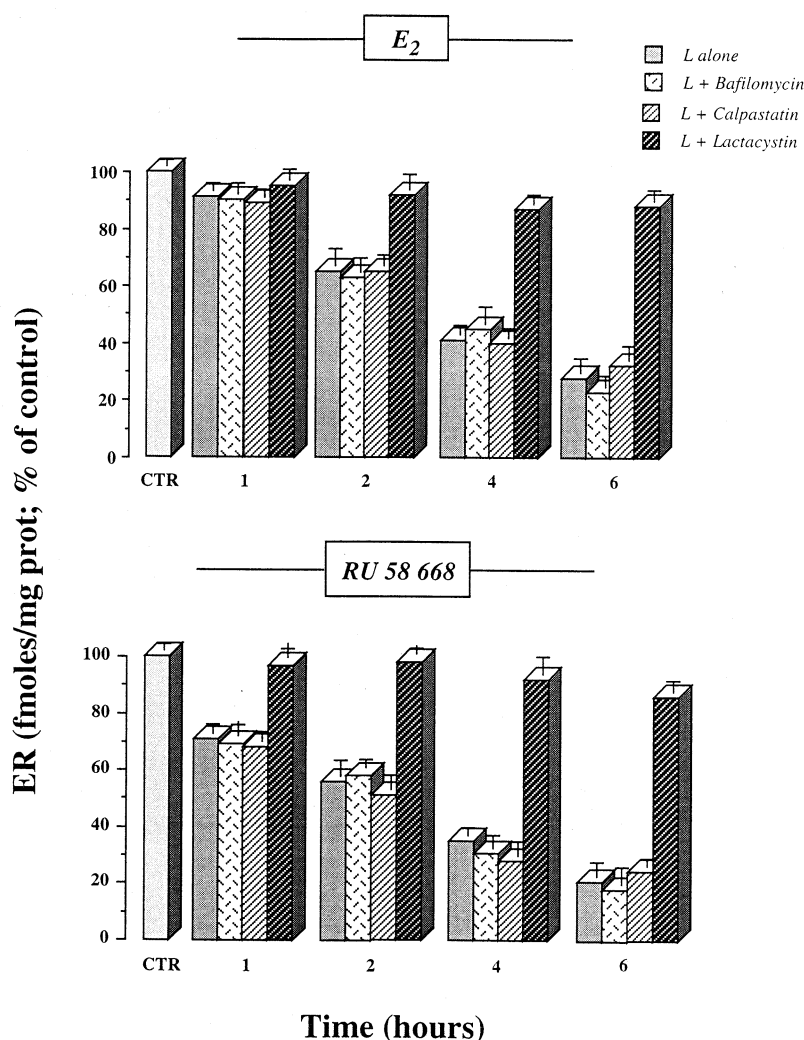


Fig. 2. Effect of proteolytic inhibitors on ligand-induced ER elimination. MCF-7 cells were incubated for 1 to 6 h either with 1 nM E_2 or 10 nM RU 58668 in the presence or absence of 10 μ M bafilomycin, 0.05 μ g/ml calpastatin or 10 μ M lactacystin (L: ligand). After removal of the medium, cells were washed, harvested, extracted and their ER contents measured by the Abbott's enzyme immunoassay (ER-EIA). Control cells were incubated without drugs. The data refer to the mean \pm S.D. of three separate experiments (CTR: 791, 816, 843 fmoles/mg protein = 100%).

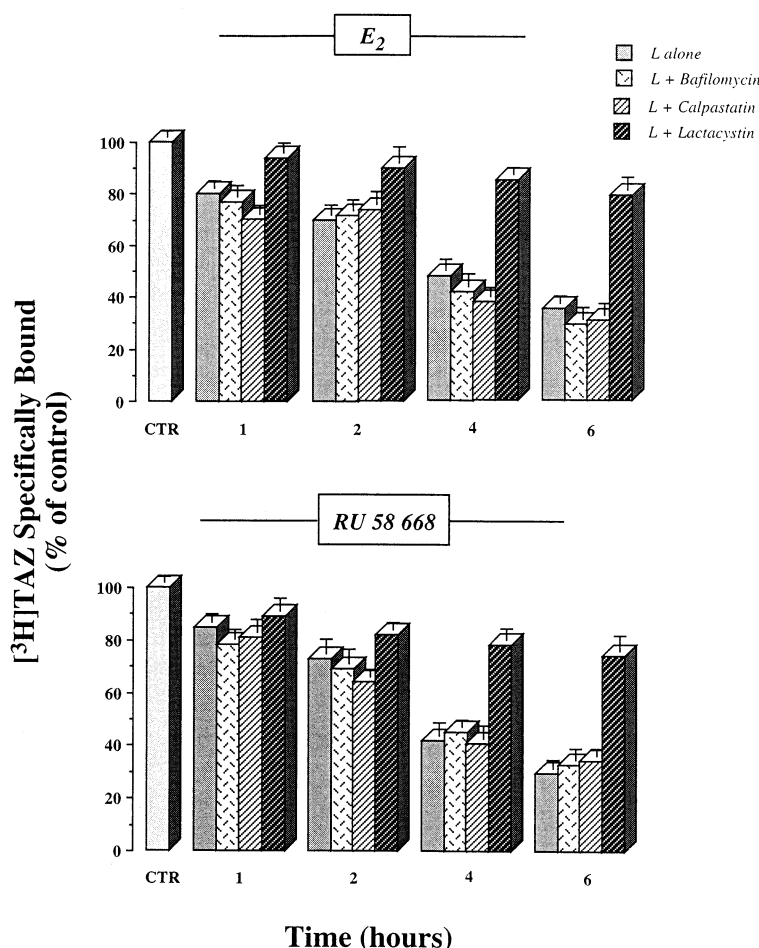


Fig. 3. Effect of proteolytic inhibitors on the ligand-induced $[^3\text{H}]\text{TAZ}$ -ER complex elimination. MCF-7 cells were labeled with 20 nM $[^3\text{H}]\text{TAZ}$ for 1 h and subsequently exposed either to 1 nM E_2 or 10 nM RU 58668 in the presence or absence of 10 μM bafilomycin, 0.05 $\mu\text{g}/\text{ml}$ calpastatin or 10 μM lactacystin for the indicated time (L: ligand). After removal of the medium, cells were washed, harvested, extracted and $[^3\text{H}]\text{TAZ}$ -ER complexes were selectively immunoadsorbed with H_{222} anti-ER monoclonal antibody and their radioactivity measured. The data refer to the mean \pm S.D. of three separate experiments (CTR: 7989, 8835, 8693 dpm = 100%).

3.2. Effects of protease inhibitors on the ligand-induced ER elimination

Since CHX and puromycin were reported to inhibit protein hydrolysis by decreasing the amounts of lysosomal enzymes [29,30], we investigated if these organelles could play a role in ER degradation specifically under E_2 stimulation. In the presence of bafilomycin, a specific inhibitor of the lysosomal proton pump [31], E_2 as well as RU 58668 maintained their ability to decrease ER concentration suggesting that lysosomal proteases were not involved in this process (Fig. 2). Hence, ER elimination seems in each case to proceed via an extralysosomal proteolytic system. The fact that ER is a substrate of calpain [13] led us to examine the involvement of the latter. The results obtained with the use of an endogenous calpain-specific inhibitor, calpastatin [32], did not support this possibility (Fig. 2). We then assessed the potential implication of the proteasome complex in ER elimination. Remarkably, lactacystin (a highly specific, irreversible, proteasome inhibitor [33]), abrogated ER elimination induced by both E_2 and RU 58668 (Fig. 2). Interestingly, ER prelabeled with $[^3\text{H}]\text{TAZ}$ before exposure to E_2 or RU 58668, and in the presence of these various proteolytic inhibitors, behaved sim-

ilarly: while lactacystin suppressed the elimination of the $[^3\text{H}]\text{TAZ}$ -ER complexes, bafilomycin and calpastatin appeared ineffective (Fig. 3). Hence, the proteasome complex appears to be always implicated in ER degradation, even when its hormone binding site is covalently labeled with $[^3\text{H}]\text{TAZ}$ and is therefore not accessible to the ligands; in such a case, elimination depends upon the association of these ligands with newly synthesized receptors [4].

3.3. Effects of protease inhibitors on the binding of $[^3\text{H}]\text{E}_2$ to ER

Assessment of the effect of protease inhibitors on the capacity of MCF-7 cells to incorporate $[^3\text{H}]\text{E}_2$ confirmed our enzyme immunological data: four lysosomal inhibitors (i.e. chloroquine, NH_4Cl , bafilomycin and monensin which all inhibit cathepsin B activity, data not shown), as well as two calpain inhibitors (i.e. calpastatin or PD 150606), failed to impede the diminution of specific $[^3\text{H}]\text{E}_2$ binding of the cells. On the contrary, two proteasomal inhibitors (i.e. lactacystin and proteasome inhibitor I) maintained the $[^3\text{H}]\text{E}_2$ -ER complexes (Fig. 4). Hence, proteasome inhibitors, while inhibiting the E_2 -induced elimination of the ER peptide, did not inter-

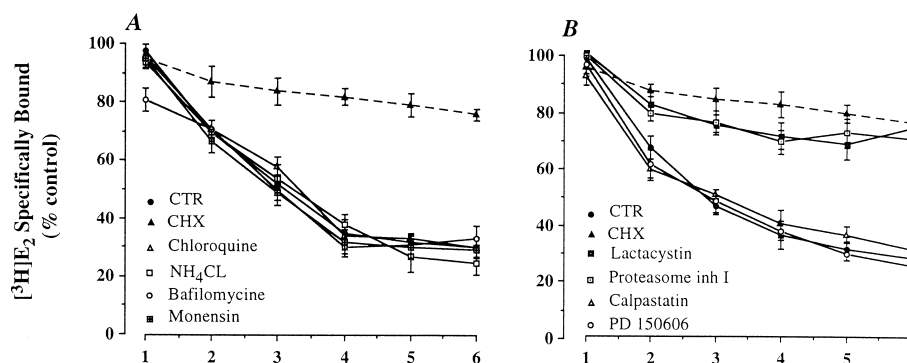


Fig. 4. Effect of proteolytic inhibitors on [³H]E₂ binding capacity. MCF-7 cells were incubated with 1 nM [³H]E₂ in the absence or presence of a given inhibitor (50 μM CHX, 100 μM chloroquine, 10 mM NH₄Cl, 10 μM bafilomycin, 10 μM monensin, 0.05 μg/ml calpastatin, 0.5 μM PD 150606, 10 μM lactacystin and 0.1 nM proteasome inhibitor I). At indicated times, specifically incorporated [³H]E₂ was extracted and measured. The data refer to the mean ± S.D. of three separate experiments (CTR: 7012, 7502, 7974 dpm = 100%).

fere with its ability to bind the hormone. As expected, control cells treated with CHX also maintained their [³H]E₂ binding capacity.

3.4. Effect of proteasome inhibitors on the ER turnover

Since the proteasome is involved in the turnover of several regulatory proteins, we investigated whether the concentration of the free (unbound) ER was similarly regulated by this proteolytic complex. To this end, MCF-7 cells were preincubated for up to 6 h with lactacystin or proteasome inhibitor I before being labeled with [³H]E₂ (1 h of incubation). Both drugs induced a slight increase of [³H]E₂ incorporation, which correlated with a receptor increase established by enzyme immunoassay (value at 6 h) (Fig. 5). Hence, the proteasome also appeared to be implicated in ER turnover.

4. Discussion

In MCF-7 cells, estrogens (E₂) and antiestrogens (RU

58668) bind to the same receptor; however, subsequent down-regulation of the corresponding complexes seems to proceed through distinct mechanisms: while CHX largely abrogates the E₂-ER elimination, it is totally ineffective on RU 58668-ER elimination [25].

All the specific lysosomal inhibitors tested here, whatever their mode of action, were ineffective in presence of both E₂ and RU 58668 indicating that the lysosomal pathway is not implicated in their induced degradation mechanism. Therefore, the specific inhibitory effect of CHX observed with E₂ would not be related to a blockade of synthesis of lysosomal proteinases, nor to the production of (a) factor(s) required for ER uptake into lysosomes.

Various transcription factors, including AP1 (c-Fos/c-Jun), ATF/CREB and AP3, are cleaved by m-calpain to produce partial digestion products [34,35]. This property raises the possibility that calpain could be involved in the turnover of transcription factors. On the other hand, most of the short-lived proteins contain one or more regions rich in proline (P),

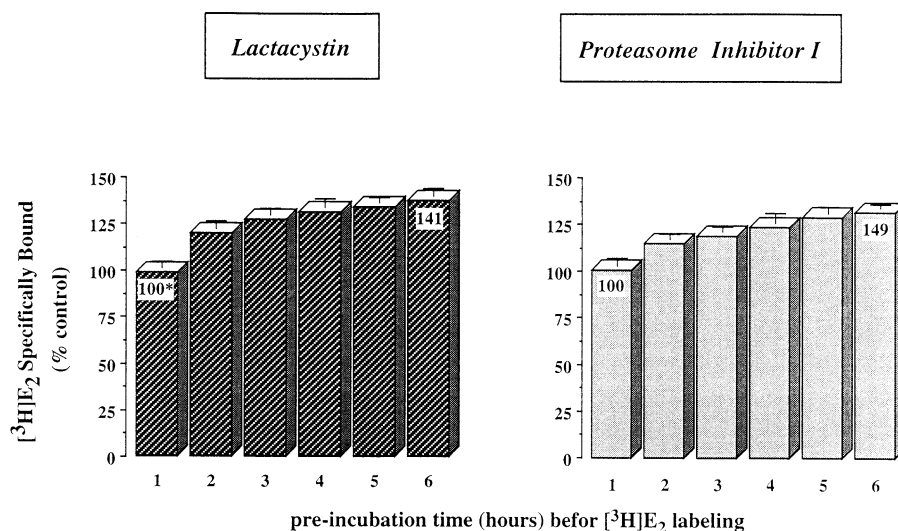


Fig. 5. Effect of proteasome inhibitors on the ER turnover rate. MCF-7 cells were incubated with either 10 μM lactacystin or 0.1 nM proteasome inhibitor I. At indicated times, medium was removed and replaced by fresh medium containing 1 nM [³H]E₂ and each inhibitor. After 1 h of incubation, specifically incorporated [³H]E₂ was extracted and measured. Control cells were maintained without inhibitors. ER contents were also determined by the Abbott's enzyme immunoassay (ER-EIA) after 6 h of exposure to each inhibitor. The data refer to the mean ± S.D. of three separate experiments (CTR: 7352, 7534, 7728 dpm = 100%). *ER-EIA values; % of control (CTR = 823, 850 fmoles/mg protein).

glutamic acid (E) and aspartic acid (D), serine (S) and threonine (T), known as PEST regions [36]. These regions are recognized by (a) specific protease(s) including calpains [37]. Computer sequence analysis of the human ER revealed a PEST sequence within its carboxyl terminus (F domain) [38] suggesting a possible influence of this region in the stability of the receptor since calpain has been reported to hydrolyse the cytosolic ER. Calpastatin, an endogenous inhibitor of calpain I as well as PD 150606, a synthetic inhibitor of calpain I and calpain II [39] were found to be ineffective in blocking ER degradation induced by both E_2 and RU 58668, which is against the implication of the calpain system in the phenomenon. In fact, this observation is in agreement with data published by Katzenellenbogen, who demonstrated that the PEST region of ER is not a feature determining its degradation rate [38]. The potential role of calpains in ER function remains unknown. One may speculate that they allow the emergence of specific cleavage ER products which accomplish functions distinct from those of the native receptor.

In our experiments, elimination of ER by E_2 or RU 58668 is always prevented by proteasome inhibitors. Hence, the proteasome appears to be the major proteolytic pathway for the estrogenic and antiestrogenic induced degradative process, raising the question of the selective inhibition by CHX. Proteasomal degradation does not only require an active multi-proteinase complex, but also a signal for substrate targeting [36,40,41]. In this regard, the best characterized mechanism is the ubiquitin system: first, ubiquitin is added to the target protein by successive enzymatic reactions, then the tagged protein is finally degraded by the proteasome [42,43]. According to this point of view, we suggest that E_2 may induce at least one or more of these enzymes, and that CHX may influence on their synthesis. This suggestion would not hold for the pure antiestrogen RU 58668 since the RU 58668-ER complex degradation appears to be independent of protein synthesis. Such ubiquitin-independent recognition may occur through direct interaction of ER with the proteasome [44], its indirect association through other factors [45], or its interaction via a more generalized ubiquitin-independent tagging mechanism (e.g. phosphorylation, methylation). On the other hand, the subunit composition of the mammalian proteasome core involves several types of subunits (14 different α - and β -like polypeptides) as well as multiple distinct catalytic centers (five types of protease activities are associated with each complex). In this regard, it has been reported that interferon- γ , which enhances antigen presentation, induces the expression of three β -subunits of the proteasome [46,47], which are incorporated into the latter in the place of homologous, normal subunits. The resulting proteasome cleaves preferentially after hydrophobic and basic residues. The possibility that E_2 acts similarly is an alternative explanation for the selective sensitivity to CHX.

Our work clearly shows that ER elimination under E_2 and RU 58668 stimulations proceeds through the proteasomal pathway. We suggest two proteasomal uptake mechanisms which differ according to the nature of the ligand: a mechanism blocked by CHX and puromycin which would contribute to the physiological autodegradation of the receptor under estrogenic stimulation, and a mechanism independent of protein synthesis, which may be implicated in the effect of antiestrogenic treatments. It now remains to identify the corresponding regulatory signals as well as the step(s) at which

CHX acts to explain its selective inhibitory effect in the presence of estrogens.

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