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PRINCIPAL INVESTIGATOR: Kenneth P. Nephew, Ph.D.
Meiyun Fan, Ph.D.
Teresa Craft

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, Indiana 46202-5167

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6. AUTHOR(S)
Kenneth P. Nephew, Ph.D.
Meiyun Fan, Ph.D.
Teresa Craft

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Indiana University
Indianapolis, Indiana 46202-5167

E-Mail: knephew@indiana.edu

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13. ABSTRACT (Maximum 200 Words)

Estrogens play important roles in both the onset and malignant progression of breast cancer. The content of estrogen receptors in breast tumors is a valuable predictor of whether a patient will respond to therapy with antiestrogens, such as tamoxifen and fulvestrant (ICI 182,780). Expression and activity of ER can be lost or impaired in antiestrogen-resistant breast cancer. The proposed studies are designed to test the overall hypothesis that the ubiquitin-like NEDD8 protein modification pathway represses estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of this pathway may prove to be a valid target for novel therapeutics. This study on mechanisms that regulate ER levels and activity are highly relevant to the development and progression breast cancer, including tumor progression to states of hormone independence and antiestrogen resistance. Thus, understanding how the estrogen receptor is regulated is an area of research critical to understanding the tissue selective pharmacology of estrogens. In addition, tamoxifen and other selective estrogen receptor modulators target the estrogen receptor, and this study is of the utmost relevance to those important therapies.

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INTRODUCTION: (Briefly, one paragraph, describe the subject, purpose and scope of the research)

Estrogen regulates diverse biological processes through estrogen receptors (ERα and ERβ) (1). Receptor levels and dynamics have a profound influence on target tissue responsiveness and sensitivity to estrogen, and receptor turnover rates provide estrogen target cells with the capacity for rapid regulation of receptor levels and thus dynamic hormone responses (2). Furthermore, several experimental results have recently demonstrated that receptor degradation is a key component of the response of cancer cells, including breast cancer cells, to antiestrogen therapy (3-5). In advanced stage breast cancers, estrogen receptor expression and activity can be lost or impaired, and the tumors are often resistant to endocrine therapies, such as the steroidal antiestrogens, ICI 182,780 and ICI 164,384 (6, 7). Our findings during the funding period have raised the intriguing possibility for a role of ubiquitin and ubiquitin-like pathways, including the NEDD8 pathway, in ERα ubiquitination and degradation and suggest that disruptions in such pathways may contribute to the development of antiestrogen-resistance in human breast cancer. This proposal will continue to test the overall hypothesis that the ubiquitin protein modification pathways repress estrogen action by facilitating degradation of ER protein. Perturbation of this pathway may prove instrumental in breast tumor progression; alternatively, activation of these pathways may prove to be a valid target for novel therapeutics.

BODY (describe the research accomplishments associated with each task outlined in the approved Statement Of Work)

To address the first task of determining the effect of Uba3 on breast cancer cell proliferation, we attempted to generate a stable breast cancer cell expressing a dominant negative Uba3 (C216S), a mutant that we had used previously to block the NEDD8 pathway (8, 9). However, we were unable to generate breast cancer cells expressing this potent mutant (data not shown). Apparently, blocking this pathway is lethal and the cells die. Thus, we will use an inducible promoter as a way to control expression of C216S levels. Those experiments are underway.

The second task of the project was to determine the molecular mechanisms of ERα corepression by the NEDD8 pathway. Toward this goal, we constructed Uba3 deletion constructs lacking one or both of the presumptive nuclear receptor interacting motifs (the NR boxes). Protein-protein interaction studies were performed, using GST-pulldown assays and x-ray crystallography studies were conducted to determine which receptor domains mediate the interactions between ERα with Uba3. We were unable to detect direct interaction of the deletion mutant constructs with estrogen receptor (data not shown). However, this could be due to important changes in protein conformation due to the removal of amino acid sequences. Thus, we have taken an alternative approach and are generating point mutations within the NR boxes. The new constructs will be examined for direct interactions with ER.

As completion of the items in task 3 was reported during the last progress period, we continued to perform further investigations into the roles of ubiquitin-like pathway NEDD8 in the responses to estradiol and antiestrogens. This was deemed a logical extension of the SOW and

within the scope of the fundamental questions underlying the SOW. Thus, the role of the ubiquitin-proteasome pathway in ERα-mediated transcriptional responses in breast cancer cells was investigated. Genetic and pharmacologic approaches were utilized to disrupt ERα ubiquitination, proteasome-mediated proteolysis and thus ERα degradation, including a dominant negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S) (8, 9), the 20S proteasome inhibitor MG132, a ubiquitin mutant with all of its lysines mutated to arginine (UbK0) (10, 11), and the partial agonist/antagonist tamoxifen. To determine the effect of blocking ERα degradation on estradiol-induced transcriptional responses, estrogen receptor-responsive reporter assays and expression of endogenous ER-target genes in MCF7 human breast cancer cells were utilized.

The results of this study are described in the attached manuscript (10), and some of the key findings are highlighted here. We show that proteasomal degradation is not essential for transcriptional activity of ERα and suggest that the ubiquitin-proteasome system functions to limit estradiol-induced transcriptional output. The results demonstrate that blocking polyubiquitination of ERα stabilizes the receptor, resulting in the prolonged expression of ERα-responsive genes (Figure 1B,C). Inhibiting the proteasome enhanced ERα transcriptional activity in MCF7 human breast cancer cells (Figure 5A,B), indicating that ERα degradation plays a key role in limiting estradiol-induced transcriptional responses in these cells. The results further suggest that in cells containing low levels of ERα, proteasome-mediated receptor degradation plays a role in limiting estradiol-induced transcriptional responsiveness (Figure 1B). While blocking ERα degradation increased the magnitude of estradiol-induced gene transcription, no effect on hormone sensitivity was observed (Figure 2). However, inhibiting the proteasome increased both the magnitude and duration of estradiol-induced expression of an ERα-target gene in breast cancer cells (Figure 5A). Overall, the data support the hypothesis that proteasome-mediated degradation of ERα serves as a means to limit the duration of estradiol signaling in receptor positive breast cancer cells. The important implication of this study is that the estradiol-induced transcriptional response is limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ERα could lead to an enhanced cellular response to estradiol in breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- Showed that inhibiting the proteasome enhances ERα transcriptional activity in MCF7 human breast cancer cells, indicating that ERα degradation plays a key role in limiting estradiol-induced transcriptional responses in these cells.
- Demonstrated that inhibiting the proteasome increased both the magnitude and duration of estradiol-induced expression of an ERα-target gene in breast cancer cells.
- Demonstrated that blocking polyubiquitination of ERα stabilizes the receptor and prolongs expression of ERα-responsive genes.
- Determined that proteasomal degradation is not essential for transcriptional activity of ERα and that the ubiquitin-proteasome system appears to function to limit estradiol-induced transcriptional output.
- The data show that the estradiol-induced transcriptional response appears to be limited by receptor degradation through the ubiquitin-proteasome system, and defects in

proteasome-mediated degradation of ERα could lead to an enhanced cellular response to estradiol in breast cancer cells.

REPORTABLE OUTCOMES (List reportable outcomes that have resulted from this research)

Manuscripts

*Fan M, Nakshatri H, **Nephew KP** Inhibiting Proteasomal Proteolysis Sustains Estrogen Receptor-α Activation. Mol Endocrinol (in press; attached) *This award is acknowledged in this publication

Presentations

1) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated estrogen receptor-α (ER) degradation in estrogen responsiveness. Abstract 4899, 94th AACR Annual Meeting of the American Association for Cancer Research (poster/discussion)
2) Fan M, Nakshatri H, **Nephew KP** 2003 The role of proteasome-mediated estrogen receptor-α (ER) degradation in estrogen responsiveness. Abstract 154; Nuclear Receptors: Steroid Sisters, Keystone Symposium, Keystone, CO
3) Fan M, Nakshatri H, **Nephew KP** 2003The role of proteasome-mediated degradation of estrogen receptor-α in estrogen-induced transcriptional response. Elwood Jensen Symposium on Nuclear Receptors and Endocrine Disorders. University of Cincinnati, Cincinnati, OH
4) Fan M, Nakshatri H, **Nephew KP** 2004. Uncoupling estrogen receptor-α transcriptional activity from receptor degradation 2nd Biannual Midwest Regional Molecular Endocrinology Conference, Indianapolis, IN (platform talk)

CONCLUSIONS

In target tissues where ERα levels are limiting, the magnitude of the response to estradiol is correlated with cellular ERα concentrations (2, 12). The ubiquitin-proteasome pathway, by modulating receptor protein turnover, could play an important role in determining cellular responses to circulating estradiol levels. Our results indicate that the magnitude and duration of estradiol-induced gene transcription are limited by proteasome-mediated degradation of ERα; therefore, it seems reasonable to speculate that defects in ERα-degradation could lead to enhanced cellular responsiveness to estrogens. In support, aberrant ERα expression and estrogen responsiveness have been linked to breast tumor pathogenesis and development (13-15), and during the previous project period we reported that blocking ERα degradation rendered breast cancer cells insensitive to the growth inhibitory effects of ICI 182,780, a potent ERα downregulator (9). We will attempt to elucidate whether defects in the ERα degradation pathway contribute to deregulated estrogen signaling in breast cancer cells and play a role in disease progression to antiestrogen resistance.

For the "so what section" (evaluates the knowledge as a scientific or medical product to also be included in the conclusion of this report), the loss of ERα degradation pathway(s) may provide a mechanism by which breast cancer cells acquire antiestrogen resistance while retaining expression of ERα. Pathways that utilize the ubiquitin-proteasome system could serve as a therapeutic targets for breast cancer.

In summary, Tasks 1 and 2 are in progress. Task 3 has been completed but extended to include further investigations into the roles of ubiquitin-like pathway NEDD8 in the responses to estradiol and antiestrogens.

List of personnel receiving pay from the research effort: Kenneth P. Nephew, Ph.D., Principal Investigator; Meiyun Fan, Ph.D., Postdoctoral Fellow; Teresa Craft, M.S., Research Associate

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10. Fan M, Nakshatri H, Nephew KP. Inhibiting proteasomal proteolysis sustains estrogen receptor-α activation Mol Endocrinol (in press)
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APPENDICES

Copy of Manuscript:

Fan M, Nakshatri H, **Nephew KP** Inhibiting Proteasomal Proteolysis Sustains Estrogen Receptor-α Activation Mol Endocrinol (In Press; see attached)

Copies of Abstracts

1) Fan M, Nakshatri H, Nephew **KP** 2003 The role of proteasome-mediated estrogen receptor-α (ER) degradation in estrogen responsiveness. Abstract 4899, 94th AACR Annual Meeting of the American Association for Cancer Research (poster/discussion)
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3) Fan M, Nakshatri H, **Nephew KP** 2004. Uncoupling estrogen receptor-α transcriptional activity from receptor degradation 2nd Biannual Midwest Regional Molecular Endocrinology Conference, Indianapolis, IN (platform talk)

Abstract 1

The role of proteasome-mediated estrogen receptor-α (ER) degradation in estrogen responsiveness.

Meiyun Fan[1], Harikrishna Nakshatri[2] and Kenneth P. Nephew[1, 2, 3]
[1]Medical Sciences, Indiana University School of Medicine, Bloomington IN 47405; [2]Department of Surgery, Indiana University School of Medicine, Indianapolis, IN 46202

The hormone estrogen plays an important role in breast cancer development and progression. The actions of estrogen are mediated primarily by ER, a short-lived, ligand-activated transcription factor. Cellular turnover of ER is mediated primarily by the 26 proteasome, yet the functional consequence of receptor down-regulation and degradation on estrogen signaling is not clear. In the present study, the effect of inhibiting the 26S proteasome on ER-mediated gene transactivation was investigated. HeLa cells were transiently transfected with an ER-responsive chloramphenicol acetyltransferase reporter gene (ERE-vitellogenin-CAT) and various doses of ER expression vector (0.1 to 5 ng pSG5-ER/10^5 cells). Twenty four hours later, cells were treated with proteasome inhibitor MG132 (1 [mu]M) for 1 h followed by treatment with 17[Beta]-estradiol (E2, 10 nM) for 24 h. ER activity was determined by measuring CAT expression. A synergistic effect of MG132 on E2-induced CAT reporter expression was observed only in cells transfected with low levels of ER (0.1 - 1 ng pSG5-ER/10^5 cells); furthermore, synergism between MG132 and E2 on ER transcriptional activity was inversely correlated with the level of ER expression. In the absence of ligand, MG132 increased ER-mediated transactivation by more than two fold. In the ER-positive human MCF7 breast cancer cell line transfected with ERE-vitellogenin-CAT, MG132 treatment increased both ligand-independent and -dependent ER transcriptional activity; however, enhancement of ER transactivation function by MG132 was less compared to HeLa cells expressing a low level of ER. Treatment of MCF7 cells with Geldanamycin (GA), an HSP90 inhibitor, caused rapid degradation of ER and decreased CAT reporter gene expression, but pretreatment with MG132 blocked GA-mediated ER down-regulation and restored ER transactivation activity. Collectively, these results suggest that by regulating ER protein levels, the 26S proteasome pathway restricts ER activity and thus cellular responsiveness to estrogen. Furthermore, in a stable MCF-7 cell line containing a disrupted NEDD8 pathway, higher steady-state levels of ER were observed and cell survival rate in the presence of the antiestrogen ICI 182,780 was greater compared to wild type MCF7 cells. Collectively, these results provide further evidence suggesting that the 26S proteasome pathway functions to restrict ER activity and consequently limit hormone responsiveness by regulating ER protein levels. Inhibition of breast cancer cell growth by ICI 182,780 is mediated in part by the ability of the drug to induce ER degradation, and our studies suggest that disruptions in the ER degradation pathway may confer cells growth advantage and provide a mechanism by which cancer cells acquire ICI 182,780 resistance.

Abstract 2

The role of proteasome-mediated estrogen receptor-α (ER) degradation in estrogen responsiveness.

Meiyun Fan1, Harikrishna Nakshatri2, and Kenneth P. Nephew1,3, 1Medical Sciences, Indiana University School of Medicine, Bloomington IN 47405; 2Department of Surgery, Department of Biochemistry and Molecular Biology, Walther Oncology Center, University School of Medicine, Indianapolis, IN 46202. 3Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202.

Estrogen receptor-alpha (ERα) is a ligand-dependent transcription factor and mediator of physiological responses of tissues to 17β-estradiol (E2). Binding of E2 to ERα rapidly downregulates receptor levels through targeted degradation by the proteasome. ERα turnover appears to be coupled to the transactivation ability of ERα, but proteasome inhibitors (e.g., MG132) interfere with the production of luciferase and β-galactosidase proteins, complicating the interpretation of studies using these reporter genes to show that inhibiting proteasome degradation inhibits ERα transcriptional activity. In the present study, the effect of inhibiting ERα degradation on receptor transcriptional activity was investigated using various ERα-responsive reporter constructs (ERE-vit-CAT, ERE-pS2-Luc) and ERα-negative HeLa cells transfected with varying amounts of ERα. Cells were treated with the proteasome inhibitor MG132, and the cellular responsiveness to E2 was examined. Proteasome inhibition enhanced E2-mediated transcriptional activity of an ERα-responsive CAT reporter. In cells transfected with low levels of ERα (0.1 - 1 ng pSG5-ER/10^5 cells) and treated with MG132 and E2, a synergistic effect on ERα activity was observed, and a time course analysis showed that MG132 treatment prolonged ERα-mediated transcription. Consistent with this finding, ERα-mediated transactivation in transfected HeLa cells was prolonged by blocking receptor ubiquitination and degradation with Ubc12C111S, a dominant negative mutant of the ubiquitin-like NEDD8 conjugation enzyme. Treatment of MCF7 breast cancer cells, which endogenously express ERα, with MG132 increased E2-induced expression of both an ERα-responsive reporter gene and an endogenous ERα-target gene, pS2. Collectively, we demonstrate that the appropriate reporter gene is necessary to determine the relationship between proteasomal degradation and ERα transcriptional activity. Moreover, proteasomal degradation is not essential for ERα transactivation function, and ERα remains functional in the absence of an intact ubiquitin-proteasome system. Finally, our study shows that proteasomal degradation plays a key role in terminating ERα-mediated transcription.

Name: Kenneth P. Nephew
Phone: 812-855-9445
Code for the meeting: J8 (Nuclear receptor: steroid sisters)
Poster session

Abstract 3

Uncoupling Estrogen Receptor-α Transcriptional Activity from Receptor Degradation

Meiyun Fan[1], Harikrishna Nakshatri[2,3], and Kenneth P. Nephew[1,3,4,]
[1]Medical Sciences, Indiana University School of Medicine, Bloomington IN 47405; [2]Department of Surgery, Department of Biochemistry and Molecular Biology, Walther Oncology Center; [3]Indiana University Cancer Center; [4]Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202.

The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; csFBS, dextran-coated charcoal-stripped fetal bovine serum; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response elements; GR, glucocorticoid receptor; luciferase, firefly luciferase; PR, progesterone receptor; Q-PCR, real-time quantitative reverse transcription-PCR; SRC, steroid receptor coactivator; Ub. Ubiquitin; Ubc, ubiquitin-conjugation enzyme; Vit, vitellogenin

Key Words: estrogen receptor, proteasome, transactivation, degradation

Abstract

Estrogen receptor-alpha (ERα) is a ligand-dependent transcription factor and mediator of physiological responses of tissues to 17β-estradiol (E2). Binding of E2 to ERα rapidly down-regulates receptor levels through targeted degradation by the proteasome. ERα turnover appears to be coupled to the transactivation ability of ERα, but the functional impact of ligand-induced ERα degradation on cellular responses to E2 has not been fully established. In the present study, the effect of blocking the ubiquitin-proteasome pathway on ERα-mediated transcriptional response was investigated. In HeLa cells transfected with ERα, blocking both receptor ubiquitination and 26S proteasome-mediated turnover of ERα markedly increased E2-induced expression of an ER-responsive reporter gene. Time course studies further demonstrated that blocking ligand-induced degradation of ERα resulted in prolonged stimulation of E2-mediated gene transcription. In breast cancer MCF7 cells containing endogenous ERα, proteasome inhibition enhanced ERα-responsive reporter gene expression and expression of endogenous ER-target genes. In addition, in estrogen responsive endometrial cancer Ishikawa cells transfected with the SRC1 coactivator, 4-hydroxytamoxifen displayed full agonist activity and stimulated ERα-mediated transcription without inducing receptor degradation. Collectively, these results demonstrate that proteasomal degradation is not essential for ERα transcriptional activity and functions to limit E2-induced transcriptional output.

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Inhibiting Proteasomal Proteolysis Sustains Estrogen Receptor-α Activation

Meiyun Fan[1], Harikrishna Nakshatri[2,3], and Kenneth P. Nephew[1,3,4,5]

[1]Medical Sciences, Indiana University School of Medicine, Bloomington, IN 47405;

[2]Department of Surgery, Department of Biochemistry and Molecular Biology, Walther Oncology Center; [3]Indiana University Cancer Center; [4]Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202.

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[5]To whom correspondence should be addressed:

Kenneth P. Nephew, Ph.D.
Medical Sciences
Indiana University School of Medicine
302 Jordan Hall
1001 E. 3rd St.
Bloomington, Indiana 47405-4401
Phone: (812) 855-9445
FAX: (812) 855-4436
E-mail:knephew@indiana.edu

The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; csFBS, dextran-coated charcoal-stripped fetal bovine serum; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response elements; GR, glucocorticoid receptor; hnRNA, heterogeneous nuclear RNA; luciferase, firefly luciferase; PR, progesterone receptor; Q-PCR, real-time quantitative reverse transcription-PCR; SRC, steroid receptor coactivator; Ub. Ubiquitin; Ubc, ubiquitin-conjugation enzyme; Vit, vitellogenin

Key Words: estrogen receptor, proteasome, transactivation, degradation

Abstract

Estrogen receptor-alpha (ERα) is a ligand-dependent transcription factor that mediates physiological responses to 17β-estradiol (E2). Ligand binding rapidly down-regulates ERα levels through proteasomal proteolysis, but the functional impact of receptor degradation on cellular responses to E2 has not been fully established. In this study, we investigated the effect of blocking the ubiquitin-proteasome pathway on ERα-mediated transcriptional responses. In HeLa cells transfected with ERα, blocking either ubiquitination or proteasomal degradation markedly increased E2-induced expression of an ER-responsive reporter. Time course studies further demonstrated that blocking ligand-induced degradation of ERα resulted in prolonged stimulation of ER-responsive gene transcription. In breast cancer MCF7 cells containing endogenous ERα, proteasome inhibition enhanced E2-induced expression of endogenous pS2 and cathepsin D. However, inhibiting the proteasome decreased expression of progesterone receptor (PR), presumably due to the heterogeneity of the PR promoter, which contains multiple regulatory elements. In addition, in endometrial cancer Ishikawa cells overexpressing coactivator SRC-1, 4-hydroxytamoxifen displayed full agonist activity and stimulated ERα-mediated transcription without inducing receptor degradation. Collectively, these results demonstrate that proteasomal degradation is not essential for ERα transcriptional activity and functions to limit E2-induced transcriptional output. The results further indicate that promoter context must be considered when evaluating the relationship between ERα transcription and proteasome inhibition. We suggest that the transcription of a gene driven predominantly by an estrogen responsive element, such as pS2, is a more reliable indicator of ERα transcription

activity than a gene like PR, which contains a complex promoter requiring cooperation between ERα and other transcription factors. **INTRODUCTION**

The actions of estrogens are mediated primarily through estrogen receptors (ERα and ERβ) (1), ligand-dependent transcription factors that interact directly with estrogen response elements (EREs) in the promoters of target genes (1). Cellular levels of ERα (2), along with a large number of receptor coregulator complexes (3), play key roles in controlling appropriate physiological responses in estrogen target tissues, such as breast and uterus. Levels of ERα mRNA and protein are regulated primarily by its cognate ligand, 17β-estradiol (E2) (4-6). E2 binding results in rapid turnover of ERα protein through the ubiquitin-proteasome pathway (7-11), which has been implicated in both the overall control of gene transcription (12-16) and transactivation function of ERα and other nuclear receptors (7, 17-24).

The ubiquitin-proteasome system consists of the 26S proteasome, a complex composed of a 20S catalytic core for protein proteolysis and two ATPase-containing 19S regulatory particles that recognize polyubiquitin-tagged substrates (25). Like many other transcription factors, stimulation of ERα transcriptional activation appears to be associated with receptor ubiquitination and proteasomal degradation (11, 26). Several proteins possessing ubiquitin ligase activity (e.g., E6AP, p300, BRCA1, and MDM2), as well as SUG1, a component of the 19S proteasome, have been shown to associate with ERα and modulate receptor signaling (27-34). These observations suggest that proteasome-mediated receptor degradation is important for ER function.

Recent studies have demonstrated that inhibiting proteasomal degradation increases transcriptional activity of many, but not all, nuclear receptors, indicating a receptor-specific

effect of proteasome inhibition (17-24). Blocking ERα turnover by a proteasome-specific inhibitor, MG132, results in decreased expression of an ERα-responsive luciferase reporter, implicating that proteasomal degradation of ERα is required for its transactivation function (7, 35). However, MG132, and other proteasome inhibitors, have recently been shown to deleteriously affect on production of a functional firefly luciferase enzyme (36), complicating the assessment of studies utilizing only ERα-responsive reporters expressing luciferase, in combination with 20S proteasome inhibitors. In addition, several studies have recently suggested that receptor degradation may not be required for ERα-mediated transcription. Frasor *et al.* reported that the partial agonist/antagonist 4-hydroxytamoxifen (4-OHT), which protects ERα from proteasomal degradation (11, 37), stimulats ER-mediated transcription of a group of genes in MCF7 cells (38). Dissociation of ERα activation from degradation has also been reported in pituitary tumor cells (39, 40).

In the present study, we investigated the role of the ubiquitin-proteasome pathway in ERα-mediated transcriptional responses. Genetic and pharmacologic approaches were utilized to disrupt ERα ubiquitination, proteasome-mediated proteolysis and thus ERα degradation, including the 20S proteasome inhibitor MG132, a dominant negative mutant of the NEDD8 conjugation enzyme (Ubc12C111S) (41, 42), a ubiquitin mutant with all of its lysines mutated to arginine (UbK0) (43), and the partial agonist/antagonist 4-OHT. To determine the effect of blocking ERα degradation on E2-induced transcriptional responses, ER-responsive reporter assays and expression of endogenous ER-target genes were utilized. The results of this study demonstrate that proteasomal degradation is not essential for transcriptional activity of ERα and

indicate that the ubiquitin-proteasome system functions to limit E2-induced transcriptional output.

RESULTS

Inhibiting the proteasome increases ERα transcriptional output

The enzymatic activity of chloramphenicol acetyltransferase (CAT), luciferase (Luc) or β-galactosidase (Gal) reporter proteins is commonly used for assessing transcriptional activity of nuclear receptors in the presence of proteasome inhibitors. Recent studies with breast cancer T47D cells revealed that proteasome inhibitors (MG132, lactacystin and proteasome inhibitor I) interfere with the production of luciferase and galactosidase proteins by a post-transcriptional mechanism, while the enzymatic activity of CAT remains unaffected (36). To verify these observations in our experimental systems, we examined the effect of MG132 on expression of these reporter enzymes from constitutively active constructs in cervical carcinoma HeLa and breast cancer MCF-7 cells. Cells were transfected with RSV-CAT, SV40-Luc or pCMV-β-gal and then treated with vehicle (DMSO) or MG132 (1 μM) for 24 h. Reporter enzyme activity was determined using standard assays for luciferase, CAT and galactosidase. Treatment of HeLa cells with MG132 had no effect on CAT activity but decreased luciferase and galactosidase activity by 80% and 30%, respectively (Fig 1A, left panel). Essentially similar results were obtained using MCF7 cells (Fig 1A, right panel). These results agree with a previous report demonstrating that proteasome inhibitors have deleterious effects on the enzymatic activities of luciferase and galactosidase reporter proteins (36).

Previously, we and others showed that E2 induces ERα degradation in transiently transfected HeLa cells and MG132 abolishes such degradation (8, 9, 42). Based on the above results, we further investigated the relationship between ERα turnover and E2-induced transcriptional response using an E2-responsive CAT reporter. HeLa cells were transiently transfected with ERE-Vit-CAT and different doses of ERα-expressing construct (0.1 – 5 ng pSG5-ERα/10^5 cells). Cells were treated with vehicle (DMSO) or MG132 (1 μM) for 1 h followed by E2 (10 nM). CAT activity was measured 24 h after E2 treatment. Basal CAT activity increased, proportional to the amount of pSG5-ERα (Fig. 1B; open bars). As expected, E2 markedly induced CAT activity (Fig. 1B; gray bars); however, treatment with MG132 plus E2 resulted in greater CAT activity, compared to E2 alone (Fig. 1B; black *vs.* gray bars). Cells treated with MG132 alone exhibited slightly higher CAT activity than the DMSO control (Fig. 1B, hatched bars). A synergistic effect of MG132 plus E2 was observed in cells transfected with lower levels of ERα (0.1 – 0.3 ng pSG5-ERα/10^5 cells). For example, the combined treatment of MG132 and E2 increased ERE-CAT activity by about 7.4-fold in cells transfected with 0.1 ng pSG5-ERα/10^5 cells, whereas MG132 or E2 alone increased ERE-CAT activity by 1.82- or 3.10-fold, respectively (Table in Fig. 2B). Immunoblot analysis showed that pretreatment with MG132 effectively blocked E2-induced ERα down-regulation in HeLa cells (Fig. 1C). Taken together, these observations demonstrate that ERα retains the capacity to activate transcription in the absence of proteasomal degradation, and blocking ERα turnover increases E2-induced transcriptional output. The results further suggest that, in cells containing low levels of ERα, proteasome-mediated receptor degradation plays a role in limiting E2-induced transcriptional responsiveness.

Effect of inhibiting the proteasome on E2 sensitivity

Based on the observation that preventing receptor protein turnover increases ERα-mediated transcription, we examined the effect of inhibiting the proteasome on hormone sensitivity. HeLa cells were transfected with ERE-Vit-CAT and pSG5-ERα, treated with DMSO or MG132 for 1 h, and then treated with various doses of E2 ($1x10^{-15}$ - $1x10^{-8}$ M). CAT activity was determined 24 h after the addition of ligand. In cells transfected with 0.3 ng (Fig. 2A) or 1 ng pSG5-ERα (Fig. 2B), a hyperbolic dose response to E2 was observed; the lowest dose of hormone that induced CAT activity was $1x10^{-11}$ M E2. Increasing ERα expression (0.3 ng *vs.* 1 ng pSG5-ERα) and pretreatment with MG132 augmented maximal CAT induction by E2, but no effect on E2 sensitivity was observed. The minimal dose of E2 required to induce CAT was $1x10^{-11}$ M under all experiment conditions, and the EC50 was not different (Fig. 2). These results demonstrate that blocking ERα degradation increases the magnitude of E2-induced gene transcription but has no effect on hormone sensitivity.

Inhibiting the proteasome extends the duration of E2-induced gene transcription

The results of the above experiments suggest that inhibiting the proteasome may extend the half-life of ligand-activated ERα and thus increase receptor transcriptional output. To test the possibility that MG132 treatment would subsequently extend the duration of an E2-induced transcriptional response, we performed a time course analysis using luciferase as a reporter protein. The half-life of CAT in mammalian cells is about 50 h (44); in contrast, luciferase has an intracellular half-life of about 3 h (44), making it well suited for performing a dynamic analysis of promoter activation. Thus, we used HeLa cells transfected with ERα and ERE-pS2-

Luc to study the effect of proteasome inhibition on E2-induced transcription in a time-dependent manner. In transfected HeLa cells, E2 induced a transient induction of luciferase activity, maximal at 6 h (Fig. 3A, closed circles). Pretreatment with MG132 decreased E2-induced luciferase expression at the early time points (1.5 h to 6 h), but markedly increased E2-induced luciferase expression from 9 to 20 h (Fig. 3A, closed triangles).

As mentioned above, MG132 can inhibit luciferase production. To determine the effect of MG132 on luciferase synthesis in general, we transfected HeLa cells with a constitutively active luciferase construct (SV40-Luc). In contrast to what we observed using ERE-pS2-Luc, MG132 consistently decreased the expression of SV40-Luc during the 20 h period (Fig 4B), excluding the possibility that MG132 enhances ERE-luc activity by stabilizing luciferase protein. To subtract the general inhibitory effect of MG132 on luciferase synthesis, at each time point shown in Fig. 3C, ERα-mediated luciferase expression in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct (Normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 x [SV40-Luc activity/SV40-Luc activity in the presence of MG132]). The adjusted results clearly demonstrate that blocking receptor degradation with MG132 increases both the magnitude and duration of E2-induced gene transcription, suggesting that the duration of gene transcription induced by E2 is limited by ERα degradation through the 26S proteasome.

Inhibiting ERα ubiquitination prolongs E2-induced gene transcription

In a previous study, we used a dominant negative mutant of the NEDD8 conjugation enzyme, Ubc12C111S, to inhibit ERα ubiquitination and degradation (42). Here we used Ubc12C111S as a means to investigate the role of ERα turnover in ERα transactivation function

and to corroborate our observations using MG132. The impact of Ubc12C111S on the time-dependent induction of a reporter gene by ERα was investigated. HeLa cells were transfected with pSG5-ERα, ERE-pS2-Luc, along with a control vector (pcDNA) or a construct expressing the mutant Ubc12 (pcDNA-Ubc12C111S). In cells transfected with pcDNA, E2 transiently induced luciferase expression, and maximal induction was observed at 5 h (Fig. 3D, closed circles). However, in cells transfected with pcDNA-Ubc12C111S, a delay in peak expression of E2-induced luciferase activity was observed (9 h; Fig. 3D, closed triangles), and luciferase expression remained elevated, even 20 h after E2 treatment. No effect of Ubc12C111S on maximal E2-induced luciferase activity was observed (Fig. 3D, closed circles *vs.* closed triangles). To confirm that the observed effect of Ubc12C111S on ERα-mediated luciferase expression was specific, luciferase activity in cells cotransfected with SV40-Luc and Ubc12C111S was assessed over time. No effect of Ubc12C111S on SV40-Luc expression was seen at 6 and 12 h post-transfection; a slight increase in luciferase expression was observed at 20 h (1.3-fold; Fig. 3E). Overall, these results demonstrate that inhibiting ERα ubiquitination prolongs ERα-mediated transcription, supporting the hypothesis that proteasome-mediated degradation of ERα serves as a means to limit the duration of E2 signaling.

Blocking polyubiquitination sustains E2-induced gene expression

To determine the effect of blocking polyubiqitination on ERα-mediated transcription, we utilized a ubiquitin mutant, UbK0, which has all of its lysines replaced by arginine. This mutant competes with endogenous ubiquitin and terminates ubiquitin chains, resulting in the accumulation of short ubiquitin conjugates that cannot be degraded efficiently by the proteasome

(43). First, we examined the effect of overexpressing UbK0 on E2-induced ERα degradation. In HeLa cells cotransfected with wild-type ubiquitin (Ub) and ERα, the level of receptor protein decreased markedly after E2 treatment (Fig. 4A), accompanied by transient E2-induced expression of an ER-responsive luciferase reporter gene (Fig. 4B, 8 h *vs.* 24 h). In contrast, cells transfected with UbK0 showed sustained E2-induced luciferase expression (Fig. 4B), and no decrease in ERα protein levels was observed (Fig. 4A). Furthermore, the effect of UbK0 on ERα-induced luciferase was specific, as UbK0 showed no effect on expression of the SV40-Luc construct (Fig. 4C). These results demonstrate that blocking polyubiquitination of ERα stabilizes the receptor, resulting in the prolonged expression of an ERα-responsive gene.

Proteasome inhibition enhances ERα-mediated transcription in MCF7 breast cancer cells

To further investigate the role of ERα degradation in receptor transactivation ability under physiologically relevant conditions, we examined the effect of inhibiting the proteasome in MCF7 breast cancer cells, which endogenously express ERα. First, we examined the effect of MG132 on ERE-Vit-CAT expression in MCF7 cells. MCF7 cells were transiently transfected with ERE-vit-CAT and then treated with DMSO or MG132 (1 μM) for 1 h prior to E2 (10 nM) treatment. CAT activity was determined 24 h after E2 treatment. A 17.8±1.7 fold increase in CAT expression was seen in MCF7 cells treated with E2, compared to the control; treatment with MG132 further increased E2-induced CAT activity to 25.6 ± 2.5 fold. Therefore, inhibiting the proteasome enhanced ERα transcriptional activity in MCF7 cells, indicating that ERα degradation plays a key role in limiting E2-induced transcriptional responses in breast cancer cells.

To determine the effect of proteasome inhibition on transcription of ERα-target genes in breast cancer cells, we pretreated MCF7 cells with MG132 and examined E2-induced pS2 gene expression. ERα regulates pS2 transcription through an imperfect palindromic ERE at position -405 to -393 of its promoter region (45); pS2 expression is considered a reliable indicator of ERα transcriptional activity (46). Time-dependent effects of MG132 on heterogeneous nuclear pS2 RNA (pS2 hnRNA) levels, which reflect the rates of pS2 gene transcription (47-50), were examined. Primers amplifying the conjoining sequence between the first intron and second exon of the pS2 gene were used, and expression of pS2 hnRNA was assessed by real-time quantitative reverse transcription-PCR (Q-PCR). After administration of E2, levels of pS2 hnRNA increased by 3 h, peaked at 12 h, and then declined by 70% during the next 8 h (Fig. 5A, gray bars). However, at all time points examined, E2-induced expression of pS2 hnRNA was markedly enhanced by pretreatment with MG132 (Fig. 5A, black *vs.* gray bars), and pS2 hnRNA levels declined only by 15% from 12 h to 20 h after the combined treatment (Fig. 5A, black bars). MG132 alone showed no effect on basal pS2 hnRNA expression (Fig. 5A, hatched bars). In agreement with what we observed with pS2 hnRNA, the combined treatment of MG132 plus E2 resulted in greater expression of pS2 mRNA after 6 h, compared to E2 treatment alone (Fig. 5B, black *vs.* gray bars); pS2 mRNA levels remained markedly elevated up to 20 h, the last time point examined (Fig. 5B, black bars). The coordinate increase in E2-induced expression of both pS2 hnRNA and pS2 mRNA by MG132 excludes the possibility that MG132 inhibits the hnRNA splicing process or stabilizes pS2 mRNA. Therefore, it seems reasonable to conclude that blocking the proteasome with MG132 enhances E2-induced pS2 transcription initiation. Together, these results demonstrate that inhibiting the proteasome increases both the magnitude and duration of E2-induced expression of the endogenous pS2 gene in breast cancer cells.

We also examined the effect of MG132 on mRNA expression of cathepsin D and progesterone receptor (PR), two well-known E2-regulated genes, in MCF7 cells. As shown in Fig. 5C, a transient increase in cathepsin D mRNA expression was observed after treatment with E2. Pretreatment with MG132 enhanced both basal and E2-induced cathepsin D expression at 3 and 6 h (Fig. 5C, black *vs.* gray bars); however, at 12 and 24 h, the effect of MG132 was no longer apparent. Treatment of MCF7 cells with E2 increased PR mRNA levels 7-fold by 3 h, and PR mRNA levels remained elevated throughout the experiment period (Fig. 5D, gray bars). MG132 pretreatment decreased E2-induced expression of PR mRNA by over 50% at all time points examined (Fig. 5D, black *vs.* gray bars), which agrees with a recent report that MG132 inhibits ERα-induced increase in PR protein levels (7). The differential effects of MG132 on these ERα-target genes demonstrate that promoter context must be considered when evaluating MG132 regulation of ERα-mediated transcription. Immunoblotting analysis showed that pretreatment with MG132 efficiently blocked E2-induced ERα down-regulation in MCF7 cells (Fig. 5E).

4-Hydroxytamoxifen stimulates ERα-mediated transcription without inducing ERα degradation

The antiestrogen 4-OHT has been shown to up-regulate ERα levels by blocking ERα degradation (37), and previous studies have shown that 4-OHT functions as an ERα agonist in Ishikawa endometrial cancer cells (51, 52). To further examine the relationship between receptor stability and ERα-mediated transcription, we stably transfected ERα-negative Ishikawa cells with ERα. The ERα(+) Ishikawa cells were then transfected with a luciferase reporter

construct containing the human C3 promoter (C3T1-Luc) and then treated with either E2 (10 nM) or 4-OHT (1μM) for 16 h. After E2 administration, a 2-fold increase in luciferase activity was observed (Fig. 6A), accompanied by a marked decrease in ERα protein level (Fig. 6B). Treatment with 4-OHT also stimulated expression of luciferase (80% of E2-stimulated luciferase expression) (Fig. 6A), but the antiestrogen did not down-regulate ERα (Fig. 6B). Thus, these results demonstrate that the partial agonist activity of 4-OHT and ERα degradation are not coupled in endometrial cancer cells. It has been reported that the steroid receptor coactivator SRC1, by stimulating transcription activity of 4-OHT liganded ERα (53), can convert 4-OHT to a full agonist. We reasoned that if receptor degradation is essential for ERα to initiate transcription, SRC1 should enhance 4-OHT-stimulated ERα transactivation activity and, in parallel, induce proteasomal degradation of 4-OHT liganded ERα. To test this reasoning, the ERα(+)Ishikawa cells were cotransfected with a construct expressing SRC1 and C3T1-Luc, and then treated with either E2 (10 nM) or 4-OHT (1μM) for 16 h. As expected, over-expressing SRC1 resulted in similar 4-OHT- and E2-stimulated ERα activity (Fig. 6A); however, 4-OHT did not induce receptor down-regulation (Fig. 6B). Thus, under these experimental conditions, 4-OHT, even when behaving as a full agonist in the presence of an increased level of SRC-1, did not induce ERα degradation. Taken together, these results demonstrate that ERα-mediated gene transactivation can be uncoupled from receptor degradation.

DISCUSSION

Like other rapidly turned-over transcription factors, engagement of ERα in transactivation is coupled to ERα degradation by the ubiquitin-proteasome pathway (7-11, 35). However, the functional impact of ERα degradation on cellular responses to E2 has not been well established. In this study, we analyzed the effect of blocking ERα degradation on E2-induced transcriptional output. We demonstrate that blocking ERα turnover prolongs the ability of ERα to transactivate target genes and increases the output of E2-induced gene transcription. We also show that 4-OHT can act as a full agonist in Ishikawa cells overexpressing SRC-1 to stimulate ERα transcriptional activity, without inducing receptor degradation. Furthermore, proteasome inhibition by MG132 increases ERα-mediated reporter gene expression, as well as expression of endogenous ERα-target genes (pS2 and cathepsin D), in MCF7 breast cancer cells. These data demonstrate that proteasomal degradation is not essential for ERα transcriptional activity; ERα remains functional after escaping ubiquitination and proteasomal proteolysis. An important implication of this study is that the E2-induced transcriptional response is limited by receptor degradation through the ubiquitin-proteasome system, and defects in proteasome-mediated degradation of ERα could lead to an enhanced cellular response to E2.

In this study, several approaches targeting different steps in ubiquitination/proteasome proteolysis were utilized to block ERα degradation. MG132 was used to inhibit ERα proteolysis by specifically blocking activity of the 20S proteasome. A dominant negative mutant (Ubc12C111S) of the NEDD8 conjugation enzyme was used to block ERα ubiquitination by inhibiting ubiquitin ligase activity (41, 42). A ubiquitin mutant with all of its lysines mutated to arginine (UbK0) was used to block ERα polyubiquitination by terminating polyubiquitin chains

(43). One concern regarding the use of these approaches is a lack of specificity, such that the observed effect on enhanced E2-induced transcriptional output could be due to stabilization of multiple regulatory proteins, in addition to ERα. However, several observations suggest that this is not the case. MG132, Ubc12C111S and UbK0 substantially enhance E2-induced, but not basal, expression of ERE-reporter genes or the endogenous pS2 gene, suggesting that the effect of these inhibitors on ERα target gene expression is hormone-dependent and thus receptor-dependent. Furthermore, a time-dependent effect on E2-induced gene transcription was observed, which agrees with the ability of these inhibitors to block ligand-induced ERα degradation. Finally, no time-dependent effect on SV40-Luc expression was observed, in contrast to ERE-Luc, suggesting that these inhibitors do not broadly affect gene transcription in a time-dependent manner. Therefore, we conclude that MG132, Ubc12C111S and UbK0 enhance E2-induced gene transcription primarily by extending the lifetime of functional ERα.

Consistent with our ERα findings, proteasome inhibition has been shown to enhance the transcriptional response mediated by other nuclear receptors, including the glucocorticoid receptor (GR) (17, 24), aryl hydrocarbon receptor (18), peroxisome proliferator-activated receptor α (19), retinoid receptors (20) and the vitamin D3 receptor (21). However, it has also been reported that MG132 decreases transcriptional activity of PR and androgen receptor (AR) (22, 23), indicating that the effect of proteasome inhibition on transcriptional activity could be receptor-specific. This is presumably due to the involvement of mechanisms other than modulation of receptor levels; for example, MG132 inhibited AR activity by eliminating androgen-induced nuclear translocation and coactivator recruitment (22, 23).

In MCF7 cells, we observed differential effects of MG132 on E2-induced transcription of endogenous pS2, cathepsin D and PR gene, suggesting that proteasome inhibition can have promoter-specific effects on gene transcription. While the reason for this is not clear, these observations raise the intriguing possibility of a differential requirement of ERα turnover in gene transcription, such that ERα degradation is required for PR transcription, but not for pS2 and cathepsin D. However, another attractive possibility is that multiple regulatory elements, other than an ERE, could be differentially regulated by proteasome inhibition; the different structures of the PR, pS2 and cathespin D promoters may favor this possibility. For endogenous genes, the effect of estrogen is usually mediated through crosstalk between the ERE and nearby regulatory elements, and there appears to be an inverse correlation between the influence of nearby elements and the "strength" of the ERE (54). The ERE sequence in pS2 promoter deviates from the consensus palindromic ERE by 1 base pair (bp) and, when isolated from surrounding sequences, is able to mediate estrogen responsiveness (45); however, for the cathepsin D promoter, although the ERE-like sequence deviates from the consensus ERE by only 2 bp, it is unable to confer estrogen regulation alone and must cooperate with other regulatory elements (54). In the case of the PR promoter, only a half-site ERE is found, and estrogen induction of PR appears to require cooperation with nearby Sp1 and AP-1 sites (55). Based on the observation that ERE-vit-CAT (Fig. 1 B) and ERE-pS2-Luc (Fig. 2) activities correlate with cellular concentrations of ERα, we suggest that ERα levels are the determining factor for the transcription activity of genes controlled exclusively by ERE. We further suggest that transcriptional activity of endogenous genes driven predominantly by an ERE (e.g., pS2) may depend upon the availability of ERα. In contrast, the level of ERα is unlikely to be the sole determining factor for the transcription of genes without a consensus ERE in their complex

promoters (e.g., PR). In support of this notion, it has been reported that E2-induced transcription of the PR gene does not parallel ERα occupancy (55). Therefore, it is possible that MG132 inhibits PR expression through other protein factors, either directly or indirectly. In this respect, when evaluating the transcriptional activity of ERα, after escaping proteasome degradation, promoter context must be considered. Based on our and others' results (50), it is plausible that the transcription rate of a gene driven predominantly by an ERE is a more reliable "readout" of ERα transcription activity than a gene containing a complex promoter requiring ERα plus other transcription factors.

Our results differ from a previous study by Reid *et al.* (35), showing that MG132 prevented recruitment of phosphorylated RNA pol II (p-Pol II) to the pS2 promoter. This is most likely due to different experimental conditions and endpoints used in the two studies. For example, Reid *et al.* used a higher dose (10 μM) and longer pretreatment (7 h) with MG132 in their study. However, under that condition, it is not clear whether the drug had any effect on p-Pol II recruitment to non-estrogen responsive promoters. In addition, although α-amantin was used to "clean" the pS2 promoter before p-Pol II recruitment analysis, it is not clear that gene transcription resumed immediately (within a 2 h period) after α-amantin treatment. Thus, whether the differential recruitment of p-Pol II, in the absence or presence of MG132 following α-amantin pretreatment, is correlated with pS2 gene transcription remains an open question. However, the observation by *Reid et al.* (35) that the 20S proteolytic subunit does not associate with the pS2 promoter in response to E2 stimulation, agrees with numerous studies showing that the 20S proteasome subunit is not required for transcription initiation and elongation (56-60).

Our observation further shows that 20S proteasome activity is not essential for ERα-mediated gene transcription.

Although the mechanism(s) by which the proteasome modulates ERα-mediated transactivation remains to be fully elucidated, chromatin immunoprecipitation assays have demonstrated that both unliganded and liganded receptors constantly cycle on and off estrogen-responsive promoters (35). MG132 appears to halt this cyclic interaction, leading to prolonged occupancy of ERα on EREs (35). The cyclic turnover of ERα could be a mechanism used by cells to prevent multiple rounds of transcription initiation from a single promoter, thus ensuring an appropriate cellular response to changes in circulating concentrations of hormone. To support this explination, recent studies of GR show that proteasome inhibition dramatically increases both the residence time of GR on its target promoter and transcriptional output (24). In addition to extending the half-life of ligand-activated ERα, other factors, such as increased cellular concentration of receptor coactivators, could contribute to the enhancement of transcription by proteasome inhibition. Several ERα coactivators, including the steroid receptor coactivator family members (SRC1, SRC2 and SRC3) and CREB-binding protein (CBP/p300), are substrates of proteasomal degradation; proteasome inhibition appears to increase cellular concentrations of these coactivators (61).

We found that blocking ERα degradation (using MG132, Ubc12C111S or UbK0) decreases E2-induced ERE-pS2-Luc expression at earlier time points (1.5 - 6 h) following E2 treatment (Fig. 3 and 4). While the reason for this is unknown, one possibility is that ubiquitination and 20S proteasome activity are required for optimal ERα activation, perhaps by facilitating the release of ERα from pre-existing corepressor complexes. In order to fully

elucidate the physiological role(s) of ubiquitination, identification of the primary ubiquitin ligase(s) for ERα, as well as the ubiquitination site(s) in this receptor, will be necessary.

In target tissues where ERα levels are limiting, the magnitude of the response to E2 is correlated with cellular ERα concentrations (2, 62). The ubiquitin-proteasome pathway, by modulating receptor protein turnover, could play an important role in determining cellular responses to circulating E2 levels. Our results indicate that both the magnitude and duration of E2-induced gene transcription are limited by proteasome-mediated degradation of ERα; therefore, it seems reasonable to speculate that defects in ERα-degradation could lead to enhanced cellular responsiveness to estrogens. In support of this possibility, it has been demonstrated that thyroid hormone and insulin, by blocking ligand-induced ERα degradation, can augment E2-stimulated cell proliferation (39, 63). Therefore, our future studies will examine the functional impact of proteasome-mediated ERα degradation on complex biological responses to estrogens, such as mammary gland development. In addition, aberrant ERα expression and estrogen responsiveness have been linked to breast tumor pathogenesis and development (64-66). Our previous studies demonstrate that blocking ERα degradation render breast cancer cells insensitive to the growth inhibitory effects of ICI 182,780, a potent ERα downregulator (42). Whether defects in the ERα degradation pathway contribute to deregulated estrogen signaling in breast cancer cells and play a role in disease progression to antiestrogen resistance remains to be elucidated.

MATERIALS AND METHODS

Plasmid Construction

The construction of pSG5-ERα(HEGO), ERE2-pS2-Luc, pcDNA-HA-Ubc12C111S, C3T1-Luc, pcDNA-SRC1, pCS2-UbK0 and ERE-vit-CAT has been described previously (43, 67, 68).

Cell Lines

The human cervical carcinoma cell line HeLa and the breast cancer cell line MCF-7 were purchased from ATCC (Manassas, VA). The ERα-negative endometrial Ishikawa cell line was kindly provided by Dr. S. Hyder (University of Missouri, Columbia). HeLa and Ishikawa cells were maintained in minimum essential medium (MEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FBS. MCF7 cells were maintained in the same medium with the addition of 6 ng/ml insulin. Prior to experiments involving hormone treatment, cells were cultured in hormone-free medium (phenol red free MEM with 3% dextran-coated charcoal-stripped FBS (csFBS)) for 3 days.

Transient Transfection and Reporter Enzyme Assays

Cells (80% confluence) were transfected with an equal amount of total plasmid DNA (adjusted by corresponding empty vectors) by using LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium. Unless stated otherwise, 24 h after transfection, cells were treated with vehicle (DMSO) or MG132 (Sigma Chemical Co.,

St. Louis, MO) for 1 h prior to E2 (Sigma) treatment. At the end of the experiment, cell lysates were prepared for reporter enzyme assays. Luciferase activity was determined using the Luciferase Assay System (Promega Corp., Madison, WI), Gal activity was determined using a chemiluminescent reporter assay (PE Applied Biosystems, Foster City, CA) and CAT activity was determined using the colorimetric CAT ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). Total cellular protein was determined by using Protein Assay Kit (Bio-Rad laboratories Inc., Hercules, CA). Reporter activities were expressed as relative light units normalized to total cellular protein.

Quantitative Real Time PCR (Q-PCR)

MCF7 cells were plated at a density of $3x10^6$ per 10-cm dish and allowed to grow in hormone-free medium for 3 days. The cells were pretreated with MG132 (5 μM) for 1 h prior to E2 (10 nM) treatment. Total RNA was prepared by a RNAeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. RNA (2 μg) was reverse-transcribed in a total volume of 40 μl containing 400 units M-MLV (New England Biolabs, Beverly, MA), 400 ng random hexamers (Promega), 80 units RNase Inhibitor and 1 mM dNTPs. The resulting cDNA was used in subsequent Q-PCR reactions, performed in 1x iQ SYBR Green Supermix (Bio-Rad) with 5 pmol forward and reverse primers. The primers used in the Q-PCR were, for pS2 mRNA: forward primer, 5'-ATACCATCGACGTCCCTCCA-3' and reverse primer, 5'-AAGCGTGTCTGAGGTGTCCG-3' (69); for pS2 hnRNA: forward primer, 5'-TTGGAGAAGGAAGCTGGATGG -3' (start position 3997, within the intron); reverse primer, 5'- ACCACAATTCTGTCTTTCACGG -3' (start position 4126, within the second exon); for PR: forward primer, 5'-TCAGTGGGCAGATGC TGTATTT-3' and reverse primer, 5'-

GCCACATGGTAAGGCATAATGA-3' (70); for cathepsin D: forward primer, 5'-GTACATGATCCCCTGTGAGAAGGT-3'; reverse primer, 5'-GGGACAGCTTGTAGCCTTTGC-3' (71); and for β-actin: forward primer, 5'-TGCGTGACATTAAGGAGAAG-3' and reverse primer, 5'-GCTCGTAGCT CTTCTCCA-3'. Q-PCR was performed in 96-well optical plates (Bio-Rad, Hercules, CA) using an iCycler system (Bio-Rad) for 40 cycles (94^0C for 10 sec, 60^0C for 40 sec), following an initial 3 min denaturation at 94^0C. The relative concentration of RNA was calculated using the ΔΔCt method according to Relative Quantitation of Gene Expression (Applied Biosystems User Bulletin) with β-actin mRNA as an internal control. Results were expressed as relative RNA levels standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1.

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FIGURE LEGENDS

Fig. 1. Proteasome inhibition enhances E2-induced CAT reporter gene expression in HeLa cells transfected with ERα.

A. Effect of proteasome inhibition by MG132 on expression of reporter enzymes from constitutively active promoters. HeLa cells (*left panel*) were plated on 12-well dishes at a density of $1x10^5$ cells/well and cultured in hormone-free medium for 3 days. The cells were transfected with 100 ng RSV-CAT, 100 ng SV40-Luc or 5 ng pCMV-β-gal using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium containing either 0.1% vehicle (DMSO) or 1 μM MG132 for 24 h. Similarly, MCF7 cells (*right panel*) were plated at a density of $1.2x10^5$ cells/well, transfected with 250 ng RSV-CAT, 250 ng SV40-Luc or 10 ng pCMV-β-gal and then treated with DMSO or MG132 for 24 h. Reporter enzyme activities were normalized against total cellular protein and expressed as the mean±SD from three independent experiments, each in triplicate. *B. Effect of MG132 on ERα-mediated CAT expression.* HeLa cells were plated in 12-well dishes at a density of $1x10^5$ cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 100 ng ERE-vit-CAT and the indicated amount of pSG5-ERα using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h. Transfected cells were treated with DMSO or MG132 (1μM) for 1 h and then treated with 10 nM E2 for 24 h. CAT activity was determined using the colorimetric CAT ELISA kit and normalized against total cellular protein. CAT activity is expressed as the mean ± SD of three independent experiments, each performed in triplicate. Fold increases in ERE-CAT in the presence of E2±MG132 are

presented in the table. *C. Effect of MG132 on E2-induced down-regulation of ERα.* HeLa cells were plated in 60-mm dishes at a density of $3x10^5$ cells/dish and cultured in hormone-free medium for 2 days. Cells were transfected with 100 ng pSG5-ERα using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed, and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1μM) for 1 h and then treated with 10 nM E2 for 8 h. Whole cell lysates were prepared and subjected to immunoblotting analysis using an anti-ERα antibody (Chemicon). GAPDH was used as a loading control.

Fig. 2. Effect of MG132 on E2 dose-dependent induction of reporter gene expression in HeLa cells.

HeLa cells were plated in 12-well dishes at a density of $1x10^5$ cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 100 ng ERE-vit-CAT and 0.3 ng (*A*) or 1 ng (*B*) of pSG5-ERα using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (1μM) for 1 h and then treated with the indicated concentration of E2 for 24 h. CAT activities were normalized against total cellular protein and expressed as mean ± SD of three independent experiments, each performed in triplicate. EC50 range was calculated with a 95% confidence.

Fig. 3. Effect of blocking ERα turnover on time-dependent induction of reporter gene expression by E2 in HeLa cells.

A. Effect of MG132 on E2-induced expression of reporter gene. HeLa cells were plated in 12-well dishes at a density of $1x10^5$ cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 250 ng ERE-pS2-Luc and 1 ng of pSG5-ERα using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h. The transfected cells were treated with DMSO or MG132 (5 μM) for 1 h and then treated with 10 nM E2 for indicated time period. Luciferase activity was determined using the Luciferase Assay System, normalized against total cellular protein. *B. Effect of MG132 on SV40-Luc expression.* HeLa cells were transfected with 100 ng SV40-Luc. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium containing either 0.1% vehicle (DMSO) or MG132 (5 μM) for the indicated time period. Luciferase activity was determined and normalized against total cellular protein. *C. Normalized ERE-Luc activities.* ERα-mediated luciferase activity in the presence of MG132 was normalized to luciferase activity from the SV40-Luc construct (Normalized ERE-Luc activity in the presence of MG132 = ERE-Luc activity in the presence of MG132 x [SV40-Luc activity/SV40-Luc activity in the presence of MG132]). *D. Effect of overexpressing Ubc12C111S on E2-induced reporter gene expression.* HeLa cells were transfected with 250 ng ERE-pS2-Luc, 1 ng of pSG5-ERα, along with 100 ng pcDNA or pcDNA-Ubc12C111S and treated with 10 nM E2 for the indicated period of time. Luc activities were normalized against total cellular protein. *E. Effect of overexpressing Ubc12C111S on SV40-Luc expression.* HeLa cells were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-Ubc12C111S or control vector pcDNA. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for

the indicated time period. Luc activities were normalized against total cellular protein. For all assays, Luc activities are expressed as mean ± SD from three independent experiments, each performed in triplicate.

Fig. 4. Ubiquitin mutant blocks ER degradation and sustained E2-induced gene expression. *A. Overexpression of UbK0 blocks E2-induced ERα degradation.* HeLa cells were plated in 60-mm dishes at a density of $3x10^5$ cells/dish and cultured in hormone-free medium for 2 days. The cells were transfected with 150 ng pSG5-ERα, along with 150 ng pcDNA-Ub or pCS2-UbK0 using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h prior to treatment with DMSO or 10 nM E2 for 8 h. Whole cell lysates were prepared and subjected to immunoblotting analysis using an anti-ERα antibody. The coomasie stained SDS-PAGE gels show that equal amounts of cell lysates were loaded. *B. Effect of UbK0 on ERα-mediated luciferase expression.* HeLa cells stably transfected with ERα were plated in 12-well dishes at a density of $1x10^5$ cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 250 ng ERE-pS2-Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0 as indicated, using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for 24 h prior to treatment with DMSO or 10 nM E2 for the indicated time period. *C. Effect of UbK0 on luciferase expression from SV40-Luc.* HeLa cells stably transfected with ERα were transfected with 100 ng SV40-Luc, along with 100 ng pcDNA-Ub or pCS2-UbK0. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for indicated time period. Luciferase activity was

normalized against total cellular protein and expressed as the mean ± SD from three independent experiments, each performed in triplicate.

Fig. 5. Effects of MG132 on ERα-mediated transcription of endogenous target genes in MCF7 cells.

MCF7 cells were plated at a density of $3x10^6$ per 10-cm dish and allowed to grow in hormone-free medium for 3 days. The cells were pretreated with MG132 (5 μM) for 1 h and then treated with 10 nM E2 for the indicated time periods. Total RNA was prepared and subjected to Q-PCR analysis to determine the expression levels of pS2 hnRNA *(A)*, pS2 mRNA *(B)*, cathepsin D mRNA *(C)* and PR mRNA *(D)*. For all Q-PCR assays, the relative levels of mRNA were normalized with β-actin mRNA and standardized such that values obtained in cells treated with vehicle (DMSO) only were set to 1. The results were expressed as mean ± SD from two independent experiments, each in duplicate. To determine the effect of MG132 on E2-induced ER degradation, MCF7 cells were treated as in *A* and subjected to whole cell lysate preparation and immunoblotting with an anti-ER antibody *(E)*. GAPDH was used as a loading control.

Fig. 6. Uncoupling of 4-OHT induced ERα activation and ERα degradation.

A. 4-OHT stimulates ERα-mediated gene expression in Ishikawa cells. Ishikawa cells stably transfected with ERα were plated in 12-well dishes at a density of $1x10^5$ cells/well and cultured in hormone-free medium for 2 days. The cells were transfected with 250 ng C3T1-Luc, along with 100 ng pcDNA or pcDNA-SRC1 using LipofectAMINE Plus Reagent. Five hours later, the DNA/LipofectAMINE mixture was removed and cells were placed in hormone-free medium for

24 h prior to treatment with 10 nM E2 or 1 μM 4-OHT for 16 h. Luciferase activity was normalized against total cellular protein and expressed as mean ± SD from three independent experiments, each performed in triplicate. *B. Effect of 4-OHT on ERα protein level.* Ishikawa cells stably transfected with ERα were plated in 60-mm dishes at a density of $3x10^5$ cells/dish and cultured in hormone-free medium for 3 days prior to treatment with 10 nM E2 or 1 μM 4-OHT for 16 h. Whole cell lysates were prepared and subjected to immunoblotting analysis using an anti-ER antibody. GAPDH was used as a loading control.

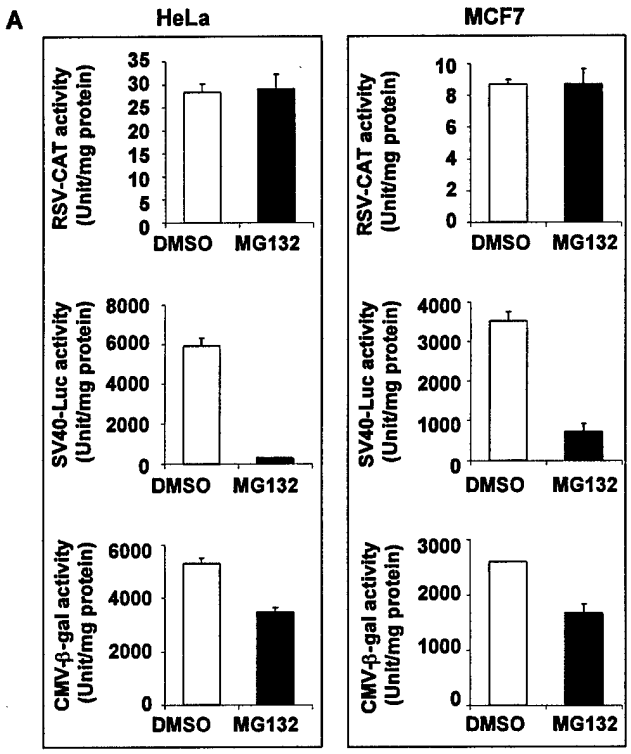
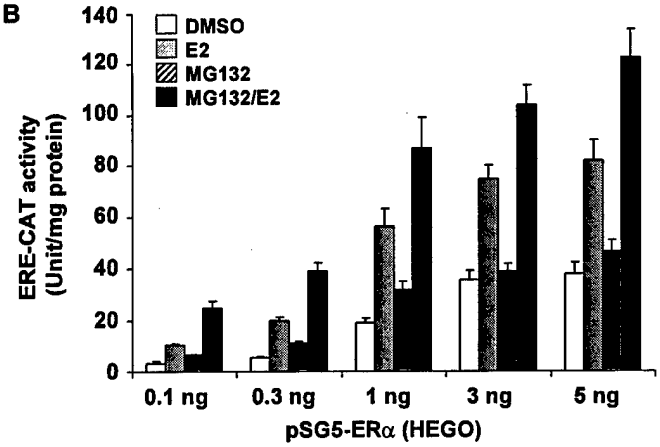

A
HeLa
MCF7
RSV-CAT activity (Unit/mg protein)
SV40-Luc activity (Unit/mg protein)
CMV-β-gal activity (Unit/mg protein)
DMSO
MG132

Fig 1A

B



The fold increases of ERE-CAT (relative to DMSO)

	pSG5-ERα				
	0.1 ng	0.3 ng	1 ng	3 ng	5 ng
DMSO	1.00	1.00	1.00	1.00	1.00
E2	3.10	3.70	3.00	2.09	2.17
MG132	1.82	2.01	1.68	1.08	1.22
MG132/E2	7.40	7.31	4.63	2.91	3.23

C

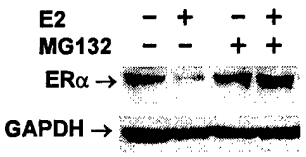


Fig 1B,C

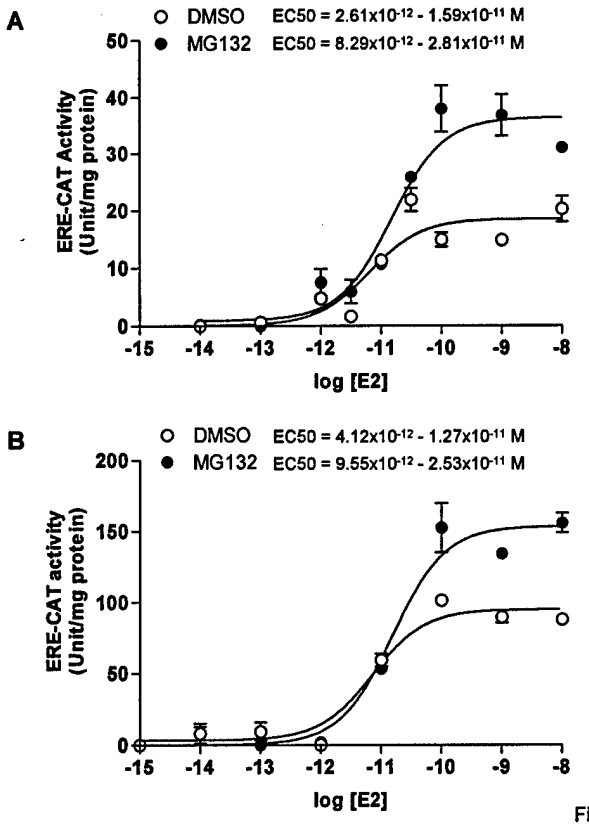

A
DMSO EC50 = 2.61x10-12 - 1.59x10-11 M
MG132 EC50 = 8.29x10-12 - 2.81x10-11 M
ERE-CAT Activity (Unit/mg protein)
log [E2]
B
DMSO EC50 = 4.12x10-12 - 1.27x10-11 M
MG132 EC50 = 9.55x10-12 - 2.53x10-11 M
ERE-CAT activity (Unit/mg protein)
log [E2]

Fig 2

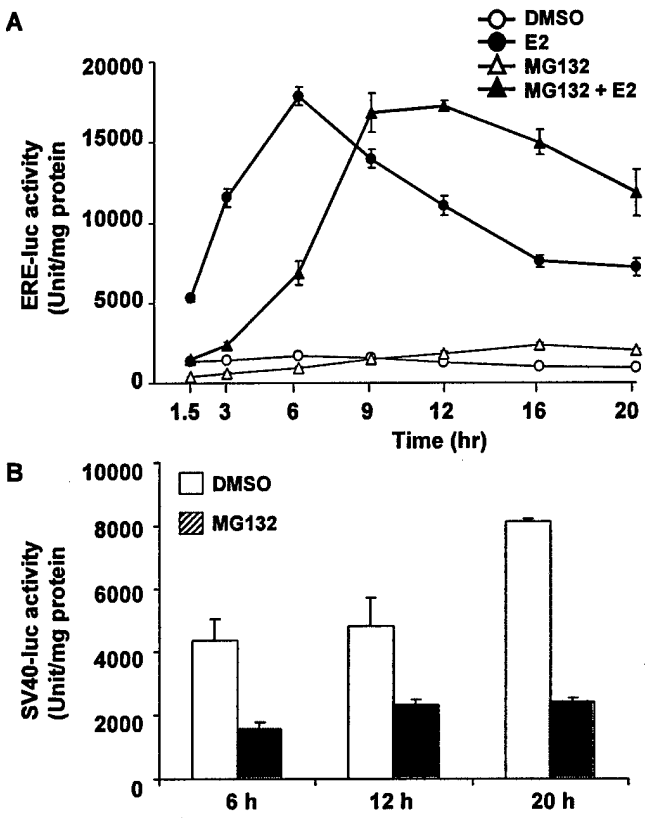

A
DMSO
E2
MG132
MG132 + E2
ERE-luc activity
(Unit/mg protein
20000
15000
10000
5000
0
1.5
3
6
9
12
16
20
Time (hr)
B
DMSO
MG132
SV40-luc activity
(Unit/mg protein
10000
8000
6000
4000
2000
0
6 h
12 h
20 h

Fig 3A,B

C

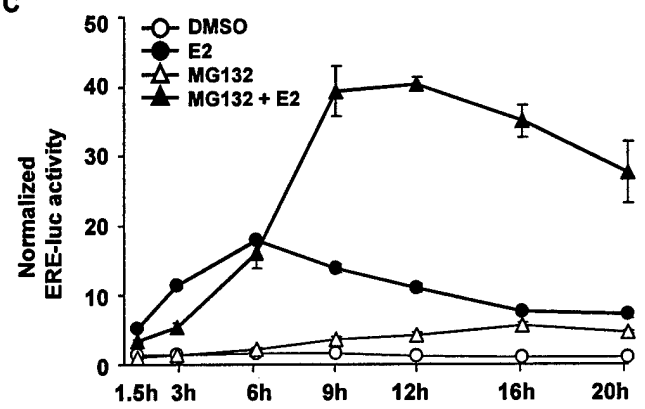
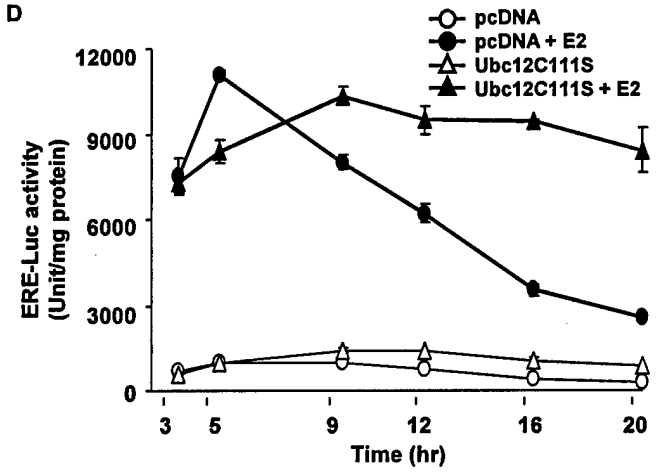

DMSO
E2
MG132
MG132 + E2
Normalized ERE-luc activity
50
40
30
20
10
0
1.5h
3h
6h
9h
12h
16h
20h

Fig 3C


D
pcDNA
pcDNA + E2
Ubc12C111S
Ubc12C111S + E2
ERE-Luc activity
(Unit/mg protein)
12000
9000
6000
3000
0
3
5
9
12
16
20
Time (hr)

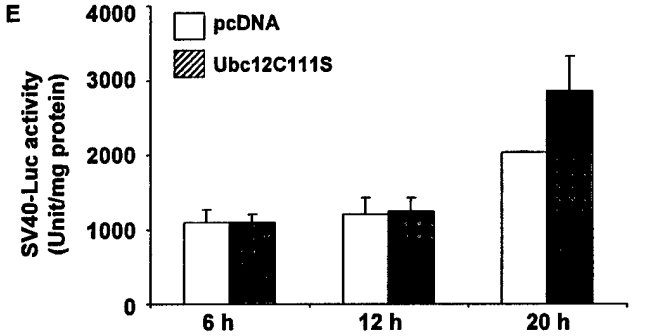

E
pcDNA
Ubc12C111S
SV40-Luc activity
(Unit/mg protein)
4000
3000
2000
1000
0
6 h
12 h
20 h

Fig 3D,E

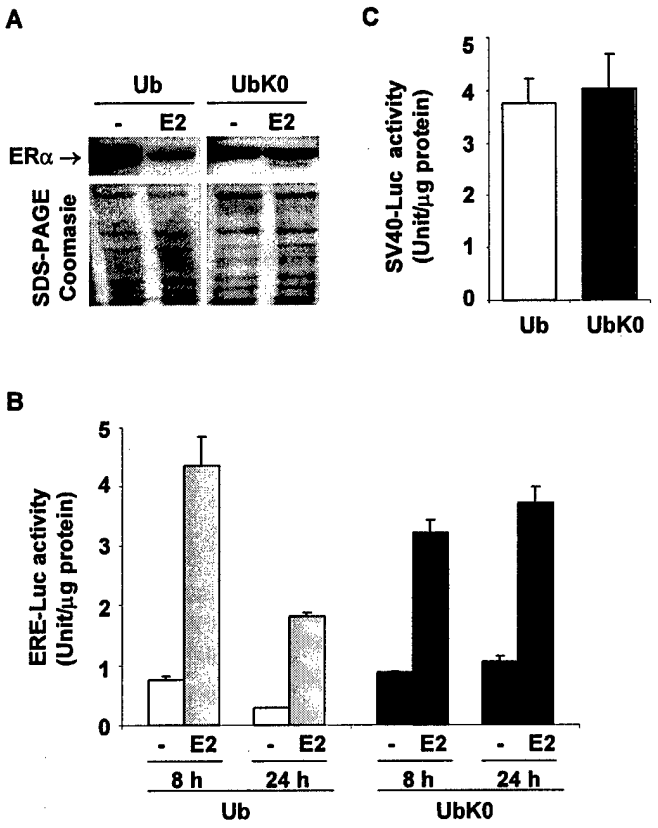

A
Ub
UbK0
- E2
- E2
ERα →
SDS-PAGE Coomasie
C
SV40-Luc activity (Unit/μg protein)
5
4
3
2
1
0
Ub
UbK0
B
ERE-Luc activity (Unit/μg protein)
5
4
3
2
1
0
- E2
- E2
- E2
- E2
8 h
24 h
8 h
24 h
Ub
UbK0

Fig 4

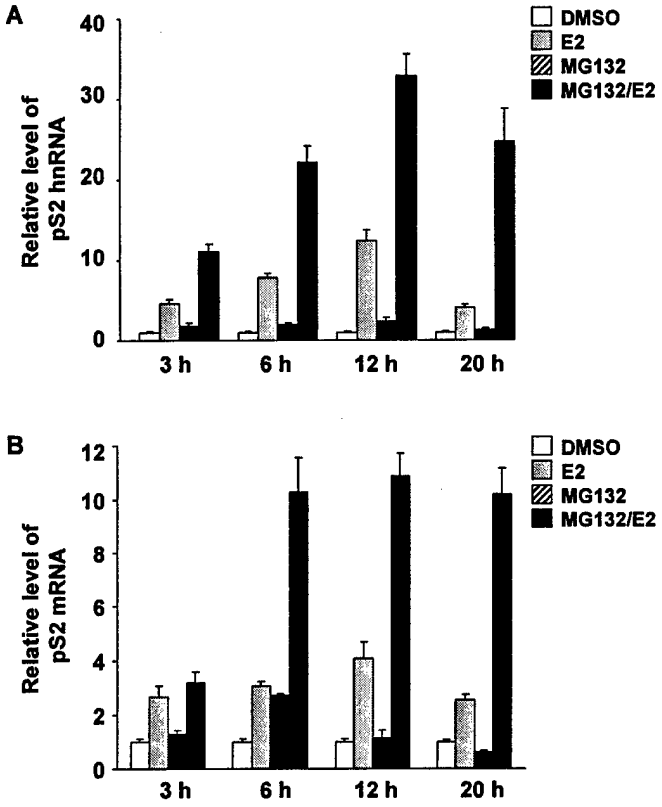

A
Relative level of pS2 hnRNA
40
30
20
10
0
3 h
6 h
12 h
20 h
DMSO
E2
MG132
MG132/E2
B
Relative level of pS2 mRNA
12
10
8
6
4
2
0
3 h
6 h
12 h
20 h
DMSO
E2
MG132
MG132/E2

Fig 5A,B

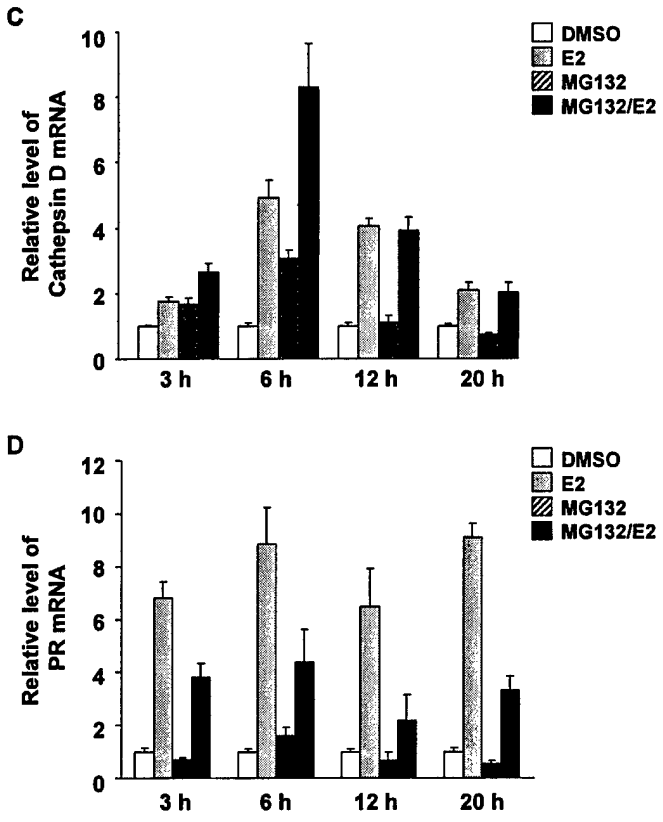

C
Relative level of Cathepsin D mRNA
10
8
6
4
2
0
3 h
6 h
12 h
20 h
DMSO
E2
MG132
MG132/E2
D
Relative level of PR mRNA
12
10
8
6
4
2
0
3 h
6 h
12 h
20 h
DMSO
E2
MG132
MG132/E2

Fig 5C,D

E

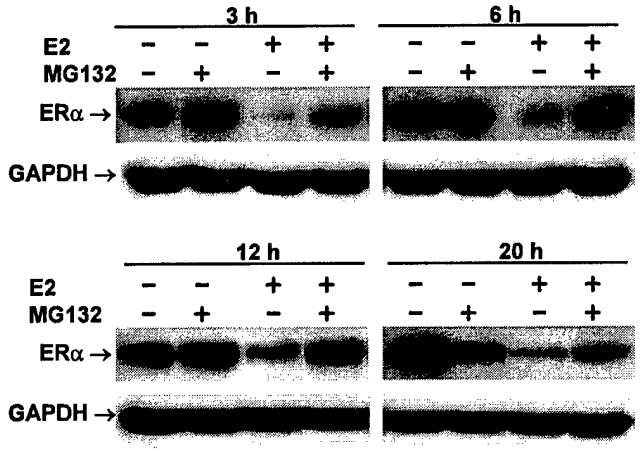


Fig 5E

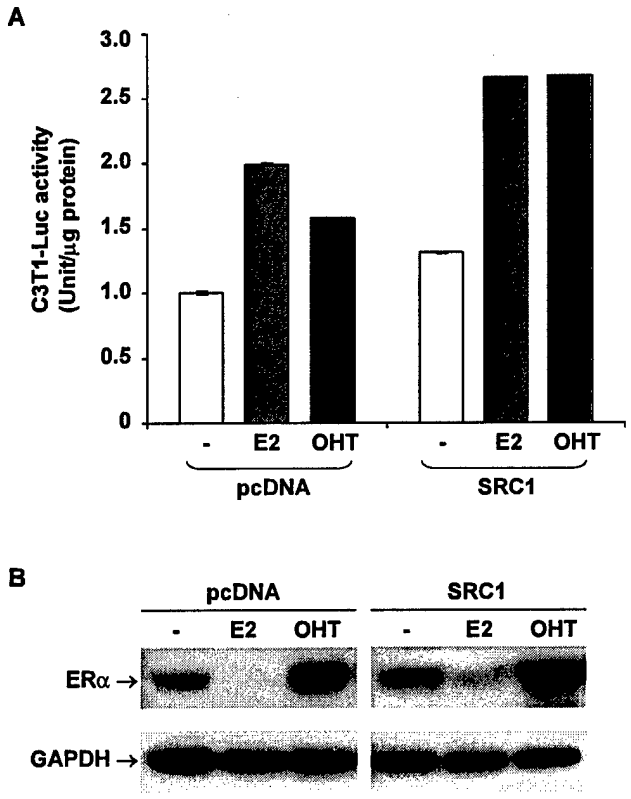

A
C3T1-Luc activity (Unit/μg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0
-
E2
OHT
-
E2
OHT
pcDNA
SRC1
B
pcDNA
SRC1
-
E2
OHT
-
E2
OHT
ERα →
GAPDH →

Fig 6