Temporal variation in estrogen receptor-α protein turnover in the presence of estrogen

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Abstract

Estrogen receptor-α (ERα) is essential in the maintenance of cellular responsiveness to estrogen in the reproductive system. It is established that ligand binding induces downregulation of ERα protein by targeting receptor for destruction by the 26S proteasome. However, ERα is preserved in cells chronically exposed to estrogen and it is unknown how receptor levels are maintained in the continued presence of the signal that induces degradation. A modified pulse-chase analysis was developed using a tet-inducible ERα expression system to determine the rate of ERα protein decay following both acute and chronic estrogen treatments. Upon initial hormone treatment, ERα half-life is shortened from 3 to 1 h. However, ERα half-life increases over time, achieving a half-life of ~6 h in 72 h of estrogen treatment. Analysis of ERα half-life in the presence and absence of proteasome inhibitor, MG132, revealed that the increased stability is due in part to a decreased rate of proteolysis. In addition, we observed a time-dependent increase in phospho-S118 ERα and showed that the half-life of the phosphomimetic ERα mutant, S118E-ER, is identical to that of wild-type receptor under conditions of chronic estrogen treatment. These data provide evidence that as cells adapt to chronic stimulation, ERα protein is stabilized due first to a decreased rate of proteolysis, and secondarily, to the accumulation of proteasome-resistant, phosphorylated form of receptor. This temporal control of proteolysis allows for the establishment of steady-state levels of receptor and provides a protective mechanism against loss of hormone responsiveness.

Journal of Molecular Endocrinology (2008) 40, 23–34

Introduction

Estrogen receptor-α (ERα) is the predominant of two ERs, α and β, that mediate the actions of estrogen in the reproductive system. It functions as a transcriptional regulator, inducing cellular responses through the regulation of gene expression. The majority of our knowledge of estrogen action comes from the analysis of ERα transcriptional activity. It has been shown that ERα-mediated transcription occurs in a well-ordered sequence of events (Shang et al. 2000, Métivier et al. 2003). These include the stable binding of receptor to DNA, recruitment of coactivator and chromatin remodeling complexes, and ultimately the recruitment of the basal transcriptional machinery. The orchestration of these events leads to gene-specific regulation with some targets being transiently induced while others, once upregulated, remaining high. The temporal regulation of gene expression mediated by ERα is critical in the timing of varied responses to estrogen, ranging from the induction of proliferation and differentiation to the maintenance of the menstrual cycle (Frasor et al. 2003, Hewitt et al. 2003).

In addition to gene expression, estrogen also induces another less well-understood cellular response, autoregulation of ERα protein. Feedback regulation of ERα by estrogen has been observed in a number of cell contexts in the reproductive system including the pituitary and mammary gland and in a number of breast and uterine cancer cell lines (Cidlowski & Muldoon 1978, Horigome et al. 1988, Read et al. 1989, Saceda et al. 1989, Kaneko et al. 1993, Keaveney et al. 1993). While negative regulation of ERα can be controlled at multiple levels, estrogen directly regulates ERα protein by targeting receptor for degradation by the 26S proteasome (Alarid et al. 1999, El Khissiin & Leclercq 1999, Nawaz et al. 1999). Proteolysis of ERα is initiated upon ligand binding (Preisler-Mashek et al. 2002), and like other targets of the 26S proteasome, ERα becomes ubiquitinated and subsequently degraded (Wijayaratne & McDonnell 2001). Mutational analysis has identified two key structural elements that are essential for estrogen-induced proteolysis, both of which are contained within transactivation domains. Specifically, substitutions at phosphorylation site, S118, in activation function-1 (AF-1), and residues I358, K362, V376, and L539 in the coactivator interaction surface of activation function-2 (AF-2) abolish ligand-induced degradation of ERα (Lonard et al. 2000, Valley et al. 2005). Beyond the
identification of these essential elements, very little is understood about the signaling events that target estrogen-bound receptor for degradation.

Previous studies have shown that estrogen-induced downregulation of ERα is an acute response to estrogen resulting in a drop of ~50% of the total receptor content within the first hours of estrogen treatment (Pink & Jordan 1996). Subsequently, cells establish a lower steady-state level of receptor. Curiously, despite the continued presence of the signal that induces both receptor proteolysis and decreased receptor synthesis, cells are not depleted of receptor. How cells maintain ERz protein levels in the presence of chronic estrogen treatment is unknown. To address this question, it is necessary to measure the half-life of ERz protein at different times following estrogen exposure. Two major strategies are used to examine the turnover rate of proteins: conventional pulse-chase analysis and pretreatment with protein synthesis inhibitors. By pulse-chase analysis, the basal half-life of ERz ranges from 3 to 5 h, depending on the cell type (Alarid 2006). Estrogen treatment significantly shortens the half-life of ERz to as little as 1 h (Alarid et al. 1999). This method is limited to the measurement of acute changes in ERz half-life due to technical issues related to deprivation of essential amino acids and pulse labeling of cells. The alternative approach is to block protein synthesis with inhibitors, such as cycloheximide, and measures protein decay by western blot analysis. The latter, however, cannot be applied to the study of ERz since protein synthesis inhibitors prevent ligand-induced turnover of the receptor (Borras et al. 1994). To overcome these challenges, we developed a modified pulse-chase method that takes advantage of tetracycline-dependent expression of an epitope-tagged ERz in a breast cancer model system. In this model, a pool of hemagglutinin (HA)-tagged ERz (ERHA) is synthesized upon treatment of cells with doxycycline (Dox; Fowler et al. 2004). Removal of Dox results in a cessation of ERHA synthesis, and ERHA protein decay can be monitored by western blot analysis. This model was utilized to examine the rate of ERz proteolysis following both short- and long-term estrogen exposures.

Materials and methods

Cell culture

A tet-inducible derivative of MCF7 breast cancer cells was engineered to express HA-tagged versions of either wild-type ERz or a phosphomimetic mutant, S118E-ERz, when treated with Dox. The tet-on MCF7 cell line overexpressing wild-type ERz (ERHA) was described previously (Fowler et al. 2004). Cells with tet-inducible expression of S118E-ERHA were created similarly. Briefly, S118E-ERHA was subcloned into the pUHD10-3 vector (Gossen et al. 1995) downstream of the tet-resistance operator. The resultant plasmid was cotransfected with a plasmid conferring puromycin resistance into a tet-responsive parental MCF7 cell line (Fowler et al. 2004). Clones were selected with puromycin and screened for induction of HA-tagged receptor in the presence of 1 μg/ml Dox by western blot analysis. Cells were maintained under standard culture conditions in a water-jacketed incubator at 37°C, 10% CO2. Growth media consisted of Dulbecco’s modified Eagle’s medium (DME; Mediatech Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin (GIBCO). For experiments involving estrogen treatment, cells were transferred to phenol red-free DMEM with 10% charcoal/dextran-stripped fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin, and 1-glutamine prior to treatment with ethanol (EtOH) or 10 nM 17β-estradiol (E2). Experiments using proteasome inhibitor consisted of a 30-min pretreatment with 10 μM MG132.

Western blot analysis

Western analysis was conducted on whole cell lysates. Following treatment, cells were washed with PBS and lysed directly in sample buffer consisting of 6.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol. Proteins were separated by gel electrophoresis on a 7-5% acrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Piscataway, NJ, USA). ERHA, ERz, and phospho-S118 ERz protein were detected with anti-HA antibody (Y-11), anti-ERz (HC-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-pS118ERz (Cell Signaling Technology Inc., Boston, MA, USA). The HC-20 antibody detects both HA-tagged and endogenous receptors. Bands were visualized by a chemiluminescence detection method (Amersham Biosciences). Receptor protein levels were measured by densitometry using Bio-Rad Quantity One Software (Bio-Rad). To quantify ERz protein levels, a standard curve was generated for each experiment using serial dilutions of control samples. Only exposures where the r values for standard curves were > 0.980 were used in the analysis of ERHA and ERz protein values. Linear regression analysis was then performed to determine the amounts of ERz protein relative to the control sample. Each point and error bars represent the mean and standard error of the three independent experiments.

Half-life measurements

Pulse-chase analysis was carried out as previously described (Alarid et al. 1999). In short, cells were
presented in Fig. 1A. Data represent the mean and standard error for three independent experiments. Changes in ERHA and endogenous ERα protein levels were determined by western blot analysis as described in Equation 1. 

$$\Sigma(tot change)_{ERα} = \Sigma(synthesis)_{ERα} + \Sigma(proteolysis)_{ERα}$$ (1)

In this equation, synthesis would be a net gain in receptors and would therefore be positive number. A net loss of receptor by proteolysis would be represented as a negative number. Treatment of cells with MG132 inhibits ERα proteolysis. Therefore, in the presence of MG132, proteolysis can be neglected. Under such conditions, 

$$\Sigma(tot change)_{ERα} = \Sigma(synthesis)_{ERα}$$ (2)

Application of this concept allows us to determine a value for ERα synthesis between time points 0–2, 2–4, 4–6, and 6–8 h in experiments conducted with proteasome inhibitor.

Equation 1 can be rearranged to allow determination of the amount of ERα proteolysis in the absence of proteasome inhibitor as follows:

$$\Sigma(proteolysis)_{ERα} = \Sigma(tot change)_{ERα} - \Sigma(synthesis)_{ERα}$$ (3)

where $\Sigma(tot change)_{ERα}$ equals the total change in ERα level measured in the absence of MG132 and $\Sigma(synthesis)_{ERα}$ equals the amount of synthesis determined from Equation 2. These calculations were used to determine the relative contribution of synthesis and proteolysis to the establishment of receptor levels upon acute and chronic treatments with estrogen. The values and calculations of ERα proteolysis over the 8-h chase for each group are described in detail in the supplementary data (Supplemental Table 1, which can be viewed online at http://jme.endocrinology-journals.org/content/vol40/issue1/).
Figure 1 ERα turnover rate at different times of pre-exposure to estrogen. (A) Schematic of the tet-inducible pulse-chase protocol for four groups pretreated for varying lengths of time with estrogen. Experiments were conducted in phenol red-free DMEM with charcoal/dextran-stripped serum. Controls included vehicle treatment of 0.1% ethanol (EtOH). Estrogen treatment (E2) consisted of 10 nM 17β-estradiol. (B) Representative western blot on whole cell lysates showing changes in ERHA protein over the chase period following removal of Dox (0, 2, 4, 6, and 8 h). (C) Levels of ERHA protein were quantified by densitometry and relative levels were determined by linear regression analysis against a standard curve generated for each blot where signal intensity at $t = 0$ was set at 1.0. Data shown represent the mean ± S.E.M. for three independent experiments. (D) The half-life of ERHA protein is plotted relative to the time of estrogen exposure. The basal half-life of receptor (EtOH-treated control) is represented by the dashed line. Data represent the mean ± S.E.M. of three independent experiments. (E) Traditional pulse-chase analysis of ERα protein levels was carried out as described in Material and methods section. Following 35S-labeling, cells were maintained in the absence (EtOH) and presence of E2 for the indicated length of time. ERα protein was immunoprecipitated, separated by gel electrophoresis, and quantified by phosphoimager analysis. Pulse-chase analysis was performed once and is consistent with previously published results (Pakdel et al. 1993, Alarid et al. 1999). (F) Western blots from C were reprobed with anti-ERα antibody (HC20) to examine endogenous ERα levels. Relative levels of ERα were determined as described in 1C. Data are presented as the mean ± S.E.M. of three independent experiments.
induction of gene expression, it is known that proteolysis of ERα is a rapid response to ligand stimulation, but it is unknown whether proteolysis is a transient or long-term response. We set out to measure the half-life of ERα protein at different time points following E2 treatment. A modified pulse-chase protocol was developed which takes advantage of a tet-inducible ERα expression system. We previously created a tet-on MCF7 cell line in which treatment with Dox results in the induction of ERα protein (Fowler et al. 2004). Since induction of ERα production is dependent on Dox, we reasoned that a treatment with Dox would generate a pool of presynthesized HA-tagged receptor akin to pulse labeling of receptor with radioactivity. After 24 h of antibiotic treatment, Dox could be washed out, and the ensuing loss of ERα protein would reflect the decay of the HA-tagged protein, analogous to protein turnover that occurs during the ‘chase’ period of a traditional pulse chase. To test this idea, cells received a ‘pulse’ of Dox for 24 h. Subsequently, Dox was removed by rigorous washing with PBS, trypsinization, and media change. Cells were then aliquoted into vehicle (EtOH) or estrogen (E2)-treated groups and collected at 2-h intervals for 8 h. To compare the effect of short- and long-term hormone treatments on ERα protein turnover, cells were pretreated for various lengths of time (0, 24, and 72 h) with estrogen or vehicle control (EtOH) prior to the pulse of Dox. A schematic of the treatment protocols for each group is shown in Fig. 1A. Changes in ERα protein levels were assessed by western blot analysis. Figure 1B shows that ERα protein levels decline at varying rates depending on the length of time of pre-exposure to estrogen. Quantification of ERα protein levels over time is shown in Fig. 1C. In the absence of estrogen (EtOH), ERα protein is lost gradually over 8 h with an approximate half-life of 2.6 h. Addition of estrogen during the chase period (E2, t=0) resulted in a more rapid turnover of ERα with a half-life of ~1 h.

For comparison, a traditional pulse-chase analysis was performed to measure the half-life of endogenous ERα in the same cell system (Fig. 1E). 35S-labeled ERα protein decayed with the basal half-life of ~2.6 h. The half-life of ERα and endogenous ERα proteins determined by the tet-inducible model and classical pulse chase respectively are comparable and are consistent with previous estimates of receptor half-life (Eckert et al. 1984, Monsma et al. 1984, Alarid et al. 1999). These results show that the turnover of ERα accurately reflects the regulation of endogenous receptor protein.

In Fig. 1D, the half-life of ERα is plotted against the time of estrogen treatment. The data reveal that the ERα protein half-life increases more than fivefold, as cells are maintained in the estrogen. Interestingly, the half-life of ERα at 24 h is similar to the basal half-life of the receptor, shown as the dashed line. This time point was of particular interest since analysis of ERα transcriptional activity by reporter gene assays is typically measured following this length of time of estrogen treatment. By 72 h, ERα protein is greatly stabilized and the half-life is ~5-6 h, close to double the basal level. Examination of endogenous ERα protein at later time points of estrogen pretreatment in Fig. 1F showed that endogenous receptor levels also fell during the chase period, though to a lesser extent than ERα.

Chronic estrogen treatment results in the establishment of lower steady-state levels of receptor in a cell. To further compare the regulation of ERα and endogenous ERα, steady-state levels of HA-tagged and endogenous receptor were determined. Cells were treated with estrogen for 0, 24, and 72 h, and the levels of HA-tagged and endogenous receptors were assessed by western blot analysis. Quantification of the relative receptor levels in Fig. 2A shows that ERα and endogenous receptor are decreased similarly by estrogen treatment. Further, there is no significant difference between groups treated for 24 and 72 h, consistent with receptor levels having reached a steady-state level with chronic hormone treatment. When the mRNA levels of ERα and endogenous receptor were examined by quantitative PCR, we found that the ERα mRNA is increased ~14-fold by Dox treatment in both absence and presence of estrogen (Fig. 2B). Withdrawal of Dox resulted in a decline in ERα but not endogenous ERα expression. These data demonstrate that the tet-induced ERα protein is regulated along with the endogenous ERα protein, and this regulation is independent of the expression level. Examination of Fig. 2B also shows that ERα mRNA expression is dependent of Dox, while endogenous ERα is unchanged by removal of Dox. The maintenance of synthesis of endogenous ERα during the chase period could offset decreases induced by protein turnover and explain the apparent increased stability of the endogenous receptor protein relative to ERα in tet-inducible pulse-chase experiments (Fig. 1F).

We reasoned that the increased stability of ERα under conditions of chronic estrogen treatment could be explained if estrogen-induced proteolysis of ERα was a transient response and had ended by 24 h. It has been established that estrogen-induced ERα degradation is mediated by the ubiquitin–proteasome pathway, and this process is effectively blocked by pharmacologic inhibitors of the 26S proteasome, such as ALLnL, lactacystin, and MG132 (Alarid et al. 1999, Nawaz et al. 1999, Reid et al. 2003). We postulated that if proteasome-dependent proteolysis of receptor had ceased, then inhibition of proteasome activity would have no effect on ERα protein turnover at later time points. Pulse-chase experiments were conducted as before in the presence of MG132 (Fig. 3A). Consistent with previous reports, proteasome inhibition abolished the basal (EtOH)
estrogen-induced degradation of ERHA (E₂, t = 0; Alarid et al. 1999, El Khissiin & Leclercq 1999, Lonard et al. 2000, Tateishi et al. 2004, Fan et al. 2005). At 24 and 72 h of estrogen exposure, inhibition of proteasome activity also prevented loss of ERHA protein and surprisingly, resulted in an increase in ERHA protein. The increase in receptor in the presence of MG132 was also observed with endogenous ERα (Fig. 3B). Results in Fig. 3C and D show that MG132 treatment did not differentially alter ERHA and ERα mRNA among the four treatment groups.

Thus, the increase in protein levels at 24 and 72 h cannot be attributed to an increase in receptor expression. It is uncertain at this point why ERα protein accumulates under these conditions. However, since MG132 inhibited the loss of receptor under all conditions, this implies that proteolysis is on-going at 24 and 72 h and hence, ERα degradation is sustained in the presence of estrogen.

Total receptor protein levels in the cell are achieved through a balance of receptor synthesis and degradation. This can be mathematically represented as the sum of ERα protein synthesis and ERα proteolysis, where positive changes represent a net gain in receptor and negative changes indicate a net loss of receptor as described in Materials and methods. Based on this simple concept, one could determine a value for either the synthesis or degradation of receptor by measuring the total change in receptor under conditions where either one of the processes was inhibited. This concept is the basis for the use of cycloheximide where the inhibition of new protein synthesis allows for the assessment of protein degradation. In our model, proteolysis is inhibited by the proteasome inhibitor, MG132, and thus the total change in receptor provides an estimate for the amount of receptor protein synthesis that occurs. Taking data from experiments described in Fig. 3, we calculated the amount of protein synthesis between 0–2, 2–4, and 4–8 h in the presence of MG132 (Supplemental Table 1). Examination of the incremental changes between each time point in the EtOH-treated group shows a net overall increase of 18 relative receptor units over the 8-h chase period (ex. 11 + 2 = 13), and this number increased to ~25 relative receptor units upon addition of estrogen. After 24 and 72 h, the overall net increase in synthesis further increased to ~64 and 102 relative receptor units respectively.

Figure 2 Coordinate regulation of ERHA and endogenous ERα protein. (A) Cells from treatment groups described in Fig. 1A were harvested immediately following the Dox pulse. Western blot analysis was performed on whole cell lysates, probing for the inducible HA tagged (ERHA) with anti-HA antibody and for endogenous receptor (ERα) with anti-ERα antibody. Relative levels of receptor were quantified as described in Materials and methods. Data shown are the mean ± S.E.M. for three independent experiments. (B) Cells were treated with 1 μg/ml Dox for 24 h followed by EtOH or 10 nM E₂ for 0, 2, 4, and 8 h. Total RNA was isolated and quantitative RT-PCR was performed using primers specific to ERHA and endogenous ERα. ERHA mRNA levels were normalized to the internal control P0 gene and calculated relative to a control sample not induced with Dox.

Given values for the amount of receptor synthesis between each collected time point, we then calculated the relative receptor units degraded by taking the total change in receptor (in the absence of inhibitors) and subtracting the amount of receptor synthesized as described in Equation 3 in Materials and methods. As an example, under basal conditions (EtOH-treated group), the measured amount of ERHA in the absence of MG132 at times 0 and 2 h are 98 and 58 respectively. Thus, the incremental change in ERHA between 0 and 2 h is 58–98 = −40 (a net loss of receptors). The measured amount of ERHA in the presence of MG132 at 0 and 2 h are 100 and 111, with a net increase of 11 receptor units (111–100 = 11). From these numbers, we can calculate the amount of receptor change due to proteolysis; total receptor – synthesized = −40 – 11 = −51. These calculations were carried out for each group of estrogen-treated samples as described in Fig. 1A, adjusting for the relative starting values of receptor (Supplemental Table 1).
Figure 4A–D shows the calculated values of ERHA protein at times 2, 4, and 8 h due to receptor proteolysis and the corresponding trend lines based on a second-order polynomial equations (correlation coefficients $r^2 > 0.9$). The first derivative of the proteolysis data trend lines for ERHA levels in Fig. 4A–D, $dy/dx$ or $d(ER\alpha\text{protein})/dt$, yields the rate of proteolysis for time points between 0 and 8 h with units of relative receptors degraded per hour (Fig. 4E). The $y$-intercept, at $t=0$ h, indicates the calculated initial rate of ERHA proteolysis for each group at the start of the chase, and the graph illustrates the change in the rate of proteolysis during the 8-h chase. Acute E2 treatment results in the highest initial rate of proteolysis of $\sim 60$ relative receptors degraded per hour. The ERHA levels at subsequent time points within the E2, $t=0$ group are not accurately described by the trend lines since receptor levels were close to the level of detection of the assay and thus the rate of proteolysis was not interpreted further. The rates of proteolysis of ERHA in the absence of estrogen and that of groups exposed to estrogen for 24 and 72 h ($E_2$, $t=24$; and $E_2$, $t=72$ respectively) were approximately equal, with estimated initial proteolysis rates of $\sim 25$ relative receptors degraded per hour at $t=0$ and ranged from 10 to 20 relative receptors degraded at $t=8$ h. These data imply that estrogen-induced proteolysis is slowed at later time points of estrogen exposure. However, the lack of difference between the rate of proteolysis at 24 and 72 h indicates that another mechanism must be invoked to explain the increased half-life of receptor between 24 and 72 h.

Figure 3 Estrogen-induced loss of ERHA protein is reversed in the absence of proteasome-mediated proteolysis. (A) Cells were treated as described in Fig. 1A. In the last 30 min of Dox induction, cells were treated with 10 µM MG132. ERHA protein was assessed by western blot analysis. Relative levels of ERHA protein were quantified as described in Materials and methods. Data are presented as the mean ± S.E.M. of three independent experiments. (B) Representative western blots for groups in A were probed with anti-ERα (HC20) antibody. The anti-ERα antibody detects both the ERHA and the endogenous ERα protein. The ERHA protein (upper band) has a slowed migration due to the presence of the C-terminal HA-tag relative to the endogenous ERα protein (lower band). A nonspecific band serves as a loading control. Total RNA was isolated from cells treated as in (A) and quantitative RT-PCR was performed using primers specific to ERHA (C) and endogenous ERα (D). ERHA and ERα mRNA levels were normalized to the internal control P0 gene and calculated relative to mRNA levels determined at $t=0$. Data represent the mean ± S.E.M. of three independent experiments.
Figure 4 Modulation of the rate of ERα proteolysis with prolonged exposure to estrogen. The change in ERHA protein as a result of proteolysis was calculated by subtracting the total change in ERHA protein for cells treated with EtOH (A), E2, t=0 (B), E2, t=−24 (C), and E2, t=−72 (D). The data used to generate the graph can be found in Supplemental Table 1. Data shown are the linear trend lines. Trend lines using second-order polynomial equations were fit to the calculated data points. The \( r^2 \) correlation coefficients for each trend line were >0.95, with the exception of E2, t=0 group with a \( r^2 \) value of 0.91. (E) The rate of proteolysis was calculated for each group by taking the first derivative of the ERHA protein trend line with respect to time, d(ERHA protein)/dt, from the proteolysis data trend lines in A–D. The resulting graph illustrates the rate of proteolysis between 0 and 8 h for each group.
Another possibility is that over time there is an accumulation of a proteasome-resistant isoform of ERα. We previously demonstrated that residue, S118, in the N-terminal AF-1 of ERα was essential for estrogen-induced proteolysis. S118 is phosphorylated in response to estrogen as well as other intracellular kinase cascades such as MAPK and TFIH/CDK7 (LeGoff et al. 1994, Kato et al. 1995, Chen et al. 2000). We examined the phosphorylation status of ERα at S118 by western blot analysis in samples from pulse-chase experiments from three groups representing vehicle-treated (EtOH), acutely estrogen-treated (E₂, t=0), and chronically estrogen-treated (E₂, t=72) cells. Samples were assessed at the beginning of the chase period and 4 h later. As is clear from Fig. 5A, ERα phosphorylation at S118 is weakly detectable in control samples and samples treated with estrogen for 4 h. However, at 72 h, there is a dramatic increase in phosphorylated receptor. Examination of ERα (lower panel) confirms that following withdrawal from Dox, ERα levels are diminished in control samples and estrogen accelerates the loss of receptor. In contrast, ERα is stable in cells pretreated with estrogen for 72 h, and the increased stability correlates with the increased level of phosphorylation of receptor at S118.

To test the causal relationship between phospho-S118 ERα with increased receptor stability under conditions of prolonged estrogen exposure, we created a stable tet-inducible MCF7 cell line that overexpresses S118E-ERHA in response to Dox. Figure 5B shows a representative screen of three colonies isolated following selection in puromycin in the absence and presence of Dox. Clone #2 showed the greatest induction in response to Dox and was chosen for further analysis. If increased levels of phospho-S118 ERα were responsible for the long half-life of receptor following 72 h of estrogen treatment, then one might predict that the S118E-ERHA would phenocopy the half-life of the wild-type receptor under the same conditions. Following the same protocol used for wild-type ERHA in Fig. 1, tet-inducible S118E-ERHA cells were pretreated with estrogen for 72 h. Cells then received a pulse of Dox and the decay of the S118E-ERHA was followed by western blot analysis using an antibody that recognizes the HA-tagged receptor. Figure 5C shows the decay of S118E-ERHA protein following removal of Dox. For comparison, the data are superimposed on results from Fig. 1B obtained in cells expressing wild-type receptor (ERHA). The turnover rates of S118E and wild-type receptor are equivalent in cells chronically exposed to estrogen. These data support the idea that phosphorylated ERα accumulates yielding a stable receptor population that can be mimicked by mutation of the phosphomimetic mutant S118E.

Discussion

The presence of ERα protein in cells is essential for conferring estrogen responsiveness, and it is through the modulation of receptor levels that cells tune their sensitivity to hormone. Estrogen concentrations can vary, but generally, cells are continuously exposed to estrogen if at low levels. Accordingly, this places a constant negative pressure to limit ERα concentrations. Yet, under conditions of chronic hormone exposure, ERα is not lost. Instead a steady-state level of receptor is achieved. Our data demonstrate that receptor protein levels are sustained in part through a combination of decreased rate of proteolysis and the accumulation of a proteasome-resistant, phosphorylated form of ERα. The coordination of these activities is regulated in a time-dependent manner. Based on our findings, we propose that like transcriptional responses to estrogen, ERα proteolysis is regulated through a temporally controlled sequence of events. Estrogen binding induces the ubiquitination of ERα, targeting a fraction of receptors for degradation by the proteasome. Simultaneously, estrogen induces the phosphorylation of receptor at S118, which can protect receptor from proteolysis, but which accounts for a small proportion of the total receptor pool. By 24 h, the rate of proteolysis is decreased, approximating that of basal conditions in the absence of estrogen. Between 24 and 72 h, the phosphorylated form of ERα has accumulated to sufficient levels to further increase the stability of the general receptor population. This gradual stabilization of receptor would confer protection against depletion of cellular receptors and contribute to the establishment of steady-state levels of ERα when cells are chronically exposed to estrogen.

The idea that changes in ERα protein levels over time can be represented as a function of synthesis and proteolysis is an application of the source-sink method used to describe and quantify dynamic systems. This method dissects dynamic systems, such as the change in ERα protein levels over time, into the sum of its sources (ERα protein synthesis) minus the sum of its sinks (ERα proteolysis). There are three possible outcomes: 1) The sum of sources is greater than the sum of sinks resulting in protein accumulation, 2) the sum of sources is less than the sum of sinks resulting in protein reduction, and 3) the sum of sources is equal to the sum of sinks resulting in steady-state levels with no change over time. While this system assumes that ERα protein has one source and one sink, protein synthesis and proteolysis respectively, the method can be used on a variety of molecules with numerous sources and sinks with each influencing total changes over time. Based on this model, we calculated the rate of proteolysis at different times of estrogen treatment. We observed a decreased rate of proteolysis by 24 and 72 h relative to initial estrogen exposure.
The data indicate that the rate of proteolysis at later time points was similar to that of the basal turnover rate of receptor in the absence of ligand. Studies by Tateishi et al. (2004) showed that the basal degradation of receptor is mediated through the ubiquitin–proteasome pathway. Further, it was demonstrated that the basal degradation pathway was distinct from estrogen-induced proteolysis and involved the ubiquitin ligase, C-terminus Hsp 70 interacting protein (CHIP). CHIP ligase is a heat shock protein-associated ligase that is found in complex with the unliganded receptor (Connell et al. 1999, Fan et al. 2005). Binding of estrogen disrupts ERα interactions with CHIP ligase and facilitates ERα interactions with other E3-ligases that ubiquitinate and bring substrate to the 26S proteasome. We tested whether ERα degradation reverted back to the basal degradation pathway by performing co-immunoprecipitations between ERα and CHIP ligase. We did not observe an increase in CHIP:ERα interactions between 2 and 24 h of estrogen treatment, though the unliganded receptor co-immunoprecipitated with CHIP ligase (data not shown). Thus, at this point, the reason behind the slowed rate of proteolysis is unclear, but it is of interest for further experimentation since it implies that the proteolytic pathway is subject to additional regulatory events over time in the presence of estrogen.

Figure 5 Increased stability of ERα under chronic estrogen conditions is related to phosphorylation of receptor on S118. (A) Cells were treated as described in Fig. 1A for groups treated acutely (t = 0) or chronically (t = 72) with estrogen. Controls included cells treated with vehicle (EtOH). Samples were collected at 0 and 4 h of the chase period. Western blots were performed on whole cell lysates and probed using anti-pS118 ERα antibody (top panel) and anti-ERα antibody (HC20; bottom panel). Shown is a representative western blot. (B) Tet-inducible MCF7 cell line was created to overexpress HA-tagged S118E-ERα (S118E-ERHA) as described in Materials and methods. Clones were treated with 1 μM Dox for 24 h and screened for expression of S118E-ERHA by western blot analysis using anti-HA primary antibody (top panel) and anti-ERα antibody (bottom panel). Shown is an example of a western blot screen of three clones. Clone 2, with the highest level of induction, was utilized in the subsequent experiments. (C) Tet-inducible pulse-chase analysis was carried out on S118E-ERHA clone 2 following 72 h of pre-exposure to estrogen (E2, t = 72). Cells were harvested at 0, 2, 4, 6, and 8 h following the removal of Dox in the continued presence of estrogen. Relative changes in S118E-ERHA were determined by quantitative western blot analysis as described in Materials and methods. For comparison, data from tet-inducible pulse-chase analysis of the wild-type ERHA from Fig. 1C is also shown.
The phosphorylation status of ERz plays an important role in the regulation of receptor protein stability. This is also the case for other nuclear receptors, including progesterone receptor, retinoic acid receptor-z, and peroxisome proliferator-activated receptor-z (Kopf et al. 2000, Lange et al. 2000, Blanquart et al. 2002). However, there is conflicting data as to whether phosphorylation stimulates or inhibits degradation. Multiple agents that activate or inhibit phosphorylation impact ERz proteolysis (Borras et al. 1994, Alarid et al. 2003, Henrich et al. 2003, Marsaud et al. 2003, Calligé et al. 2005). Curcumin, a broad spectrum kinase inhibitor, diminishes ERz phosphorylation and inhibits ERz proteolysis (Calligé et al. 2005). Like curcumin, okadaic acid inhibits ERz degradation; however, in contrast, okadaic acid increases ERz phosphorylation (Borras et al. 1994). Other examples show that inhibition of the protein kinase C (PKC) pathway prevents or has no effect on ERz degradation, while inhibition of PKA or MAPK accelerates receptor turnover (Marsaud et al. 2003). TPA, 12-O-tetradecanoylphorbol-13 acetate, an upstream activator of PKC, and MAPK have previously been shown to phosphorylate ERz on S118 (LeGoff et al. 1994, Kato et al. 1995). Mutational analysis from our laboratory also showed that mutation of S118 to either alanine or glutamic acid results in stabilization of ERz in the presence of estrogen (Valley et al. 2005). Thus, the relationship between phosphorylation of ERz and proteolysis is complex and not well defined. The studies presented here show that long-term estrogen treatment results in an increase in ERz half-life that correlates with the increase in phospho-S118 ERz. We also demonstrate that the phosphomimetic mutant, S118E, has a long half-life which mirrors that of the wild-type receptor under conditions of chronic estrogen treatment. Together, these data suggest that ERz is stabilized by phosphorylation on S118, but this effect was observed in the context of chronic estrogen treatment. Thus, whether phosphorylation facilitates or inhibits proteolysis appears to be dependent on both cell context and time.

The dynamic control of cellular ERz concentration is physiologically important. Negative regulation of ERz in the presence of estrogen is critical in limiting cellular response to hormone. It has been demonstrated in vitro, in vivo, and in clinical studies that persistent high levels of ERz have the potential to alter receptor transcriptional function and are associated with pathologies including breast cancer and infertility (Lessey et al. 1989, Khan et al. 1999, Frech et al. 2005, Fowler et al. 2006). Equally important is the requirement to sustain receptor levels to maintain sensitivity to changes in the endocrine environment. The mechanisms that control fluctuating levels of ERz protein represent a balance between those that increase receptor protein synthesis and those that degrade it. Here, we show that the estrogen-dependent proteolytic control of ERz is itself subject to regulation by factors that vary with time. Under steady-state conditions, ERz is a relatively stable protein with a constant slow rate of proteolysis. This raises interesting questions about the potential role of protein stability in the control of specific ERz functions but also emphasizes the likelihood that such roles, once identified, may be related to temporal responses to estrogen.

Acknowledgements

This manuscript is dedicated to Dr. Jack Gorksi, whose input was invaluable and who we dearly miss. Also, we thank Brian Kenealy for the technical support. This work is funded by a grant from NIH to ETA, DK 64034. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 13 November 2007

Accepted 14 November 2007

Made available online as an Accepted Preprint

14 November 2007

Journal of Molecular Endocrinology (2008) 40, 23–34

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