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Ligand-independent and agonist-mediated degradation of estrogen receptor-α in breast carcinoma cells: evidence for distinct degradative pathways

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Abstract

Molecular chaperones and co-chaperones, such as heat-shock proteins (Hsp's), play a pivotal role in the adequate folding and the stability of steroid hormone receptors. As shown by immunofluorescence staining and immunoblot analysis, the Hsp90 inhibitor radicicol induced a rapid (within hours) depletion of estrogen receptor-α (ER) in MCF-7 and IBEP-2 breast carcinoma cells. Inhibition of proteasomes (MG-132, LLnL) or of protein synthesis (cycloheximide), which both suppressed E_2-induced downregulation of ER, failed to modify ER degradation caused by radicicol. On the other hand, partial antiestrogens, such as hydroxytamoxifen (a triphenylethylene) and LY 117,018 (a benzothiophene) stabilized ER, making it immune to radicicol-induced degradation. Furthermore, radicicol did not interfere with ER upregulation induced by hydroxytamoxifen. Thus, the current study points to possible variation in the mechanism/pathway of ER breakdown. Besides, the protective effect of partial antiestrogens suggests that ER stability is only compromized by Hsp90 disruption when the receptor is in its native, unliganded form.

Keywords: Geldanamycin; Hsp90; IBEP-2; LLnL; MCF-7; MG-132; Proteasomes; Radicicol; Raloxifene; Tamoxifen

1. Introduction

Estrogens are essential for the development, the differentiation and the function of the female reproductive system. They also act as regulators in other tissues, promoting bone formation, exerting a wide variety of effects on the cardiovascular apparatus and maybe preventing age-related neurodegenerative damage in the central nervous system. On the other hand, estrogens have also been known for long to represent a major risk factor in the etiology of breast carcinoma (Key et al., 2001). According to recent estimates, breast tumors exhibit estrogen receptors (see below) in more than 70% of cases (Colditz et al., 2004). Based on numerous clinical studies, there is a general consensus concerning the benefits afforded by estrogen antagonists (antiestrogens) for the treatment of breast cancer (Early Breast Cancer Trialists Collaborative Group, 1998; Cosman and Lindsay, 1999).

Like other steroid hormones, estrogens exert their effects on target cells by interacting with cognate receptors which function as ligand-modulated transcription factors. Although two estrogen receptor isoforms (α and β) have been identified in mammals (including humans), the predominant form expressed in breast carcinoma cells is estrogen receptor α (henceforth referred to as ER). Hence, ER is the receptor involved in estrogen-mediated signalling in breast cancer cells and stands as a driving force in the development

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of estrogen-sensitive breast neoplasms (Ciocca and Fanelli, 1997).

Steroid hormone receptors, such as ER must interact with a variety of partner proteins in order to get "activated" and assume their function in hormone-mediated signalling (Pratt and Toft, 1997a). Thus native, unliganded ER occurs in complexes with the chaperone Hsp 90 and cochaperones (p23 and immunophilins) (Richter and Buchner, 2001). Chaperone association with ER contributes to maintaining receptor stability. Hsp 90 is an adenine nucleotide binding protein endowed with ATPase activity (Panaretou et al., 1998). It is only active in the ATP-bound state. Substances like geldanamycin (a benzoquinone ansamycin) and radicicol (a macrocyclic antibiotic) act as competitive inhibitors for the nucleotide binding pocket and disrupt Hsp 90 activity by mimicking its ADP-bound state (Scheibel and Buchner, 1998; Schulte et al., 1999; Piper, 2001; Neckers, 2002). This results in the degradation of ER as well as that of other Hsp 90 client proteins involved in signal transduction (Maloney and Workman, 2002).

Since steroid receptors represent a limiting factor with regard to hormone signalling (Vanderbilt et al., 1987), ER level is a critical determinant of the magnitude of the cell response to estrogenic stimulation (Webb et al., 1992). ER concentration in cells results from a dynamic balance between ER synthesis and breakdown, the latter process occurring essentially in proteasomes (Alarid et al., 1999; El Khissiin and Leclercq, 1999; Nawaz et al., 1999). Ligand binding modifies ER level mostly by altering the rate of receptor degradation. Thus, estrogen agonists (including physiological estrogens), as well as steroidal (so-called "pure") antiestrogens, have been reported to enhance ER breakdown, causing thereby a decrease in ER level (downregulation) (Dauvois et al., 1992; Wijayaratne and McDonnell, 2001). By contrast, "partial" antiestrogens, such as tamoxifen (and in particular its metabolite 4-hydroxy-tamoxifen, OH-Tam) tend to provoke ER accumulation (upregulation), at least in MCF-7 cells and related breast cancer cell lines (Gyling and Leclercq, 1990; El Khissiin et al., 2000; Wijayaratne and McDonnell, 2001; Journé et al., 2004). In a recent study (Laïos et al., 2003), we demonstrated that this upregulation is mostly, if not exclusively due to an impairment of ER degradation.

In the present study, we examined ER degradation resulting from Hsp90 inhibition and we found evidence that it might differ from ER downregulation due to agonist binding. Besides, our observations reveal that partial antiestrogens can prevent ER degradation due to Hsp90 inactivation.

2. Materials and methods

2.1. Drugs and inhibitors

17β-estradiol (E_2) and 4-hydroxytamoxifen (OH-Tam) were obtained from Calbiochem-Novabiochem (La Jolla, CA). MG-132 (carboxybenzoxy-Leu-Leu-leucinal) and cycloheximide were supplied by A.G. Scientific (San Diego, CA). Radicicol, geldanamycin and LLnL (*N*-acetyl-Leu-Leu-norleucinal) came from Sigma-Aldrich (St. Louis, MO).The raloxifene analog LY 117,018 (Schafer et al., 2001) was a kind gift from Eli Lilly & Co. (Indianapolis, IN). Stock solutions of these compounds were prepared at least 1000-fold more concentrated in ethanol and stored at −20 °C.

2.2. Cell culture

MCF-7 breast carcinoma cell line (Soule et al., 1973) (ATCC no. HTB22) was originally obtained in 1977 from the Michigan Cancer Foundation (Detroit, MI). Breast cancer cell line IBEP-2, established 6 years ago (Siwek et al., 1998), has been characterized in a recent publication from our group (Journé et al., 2004). Routine cell propagation and experimental studies were carried out at 37 °C, in a cell incubator with humid atmosphere at 5% CO_2. Unless specified otherwise, cells were cultured in T-flasks containing Dulbecco's Modified Essential Medium (DMEM, BioWhittaker Europe, Verviers, Belgium) supplemented with Phenol Red, 10% fetal bovine serum (FBS, HyClone, Logan, Utah), 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (DMEM-FBS) (supplements from BioWhittaker or Gibco™ Invitrogen, Merelbeke, Belgium). Cells were passed once or twice a week, with a renewal of the medium every 2 days. For subculture and cell plating prior to immunofluorescence, the monolayers were rinsed with Dulbecco's phosphate buffered saline (DPBS) and cells were dislodged from the vessel bottom by treatment with trypsin–EDTA solution. After vigorous pipetting, concentrations of cells in suspension were determined in an electronic cell counter (model Z1 Coulter counter). For routine culture maintenance, cells were plated in 75 or 25 cm^2 T-flasks at a density of 5000 cells/cm^2. Before treatment with drugs and inhibitors, Phenol-Red-free DMEM supplemented with 10% charcoal-stripped FBS (HyClone, Logan, Utah), 25 mM HEPES and 2 mM L-glutamine (estrogen-free medium, EFM) was substituted for DMEM-FBS and cells were grown in this medium for a minimum of 2 days.

2.3. Immunofluorescence studies

MCF-7 or IBEP-2 cells in EFM were plated at densities of 10^4 cells/cm^2 on sterile round glass coverslips in 12-well dishes. Two days after seeding, cells were fed fresh EFM containing E_2, estrogen antagonists and/or inhibitors at the concentrations indicated in Section 3. After the treatment duration specified in Section 3, cell monolayers were rinsed with DPBS and fixed for 15 min with 4% paraformaldehyde (PAF) in DPBS or with ice-cold Carnoy's mixture. After fixation, PAF or Carnoy's mixture were changed for DPBS or 70% ethanol, respectively, where the cell cultures were kept

at 4 °C until immunostaining which was performed within the next 20 h.

Demonstration of ER by immunofluorescence was achieved as detailed in a previous publication (Brohée et al., 2000). In short, cell monolayers were rinsed several times with phosphate buffered saline (PBS, 0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.12 M NaCl, pH 7.2). In the case of PAF-fixed cells, 0.1% Triton X-100 was included in buffer used for incubations and rinses, in order to ensure cell permeabilization. Before exposure to primary antibodies, cells were preincubated for 20 min. in PBS containing 5% normal goat serum (PBS-NGS) and 0.05 M NH_4Cl to prevent non specific adsorption of immunoglobulins. Several antibodies were utilized as primary reagents for ER immunofluorescence staining. Thus, we used rabbit polyclonal antibodies raised against different parts of human ER-α: H-184 (residues 2–185 encompassing the A/B domain at the aminoterminus), G-20 (residues 281–300 in the D domain) and HC-20 (residues 576–595 in the F domain at the carboxyterminus) (all three reagents from Santa Cruz Biotechnology, Santa Cruz, CA). In addition, ER was also demonstrated by the use of mouse monoclonal antibodies from clones B403 (AER308) (Abcam Limited, Cambridge, UK) and 6F11 (Novocastra Laboratories, Newcastle upon Tyne, UK). Cells were exposed for 60 min to one of the primary antibodies diluted 1:50 in PBS-NGS. Thereafter, the cell preparations were incubated for 30 min in presence of a dextran polymer conjugated with both peroxidase and antibodies raised against rabbit or mouse immunoglobulins (EnVision™, Dakopatts, Glostrup, Denmark). The next step consisted in a 30 min incubation with rabbit anti-peroxidase antiserum (Laboratory of Hormonology, Marloie, Belgium), followed by biotinylated swine anti-rabbit immunoglobulins antibodies (Dakopatts) for a further 30 min. Immunolabeling was completed by exposing cells for 30 min to Texas Red-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After final rinses in PBS, the coverslips were mounted on glass slides using commercial antifading medium (Vectashield®, Vector Laboratories). Negative controls were produced by omitting the primary antibody. This modification resulted in a virtual disappearance of the signal.

The cell preparations were examined on a Leitz Orthoplan microscope equipped with a Ploem system for epi-illumination. Excitation wavelength of 596 nm and emission wavelength of 615 nm were used for the observation of Texas Red fluorescence. The appearance of immunostained cell preparation was documented by using a PC-driven digital camera (Leica DC 300F, Leica Microsystems AG, Heerbrugg, Switzerland). Microscopic fields were digitalized thanks to a software specifically designed for image acquisition and storage (Leica IM 50). Image adjustment and printing were achieved with appropriate softwares (Corel PHOTO-PAINT™ and CorelDRAW™, Corel Corporation, Ottawa, Ont., Canada).

2.4. Western blot analysis

MCF-7 cells were plated in 60 cm^2 petri dishes at a density of 10^4 cells/cm^2 in EFM and cultured for 24 h. They were subsequently incubated with 10^{-5} M MG132, 10^{-4} M LLnL or vehicle for 1 h and then exposed to 2×10^{-6} M radicicol, 10^{-9} M E_2, 10^{-7} M 4-OH-TAM, 10^{-7} M LY 117,018, 10^{-5} M MG132 and 10^{-4} M LLnL, alone or in combination, for 6 h as specified in Section 3. Cell monolayers were rinsed twice with TBS (50 mM Tris–HCl pH 7.5 and 150 mM NaCl) and harvested using 500 μl lysis buffer (TBS containing 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 0.1 mM Na_3VO_4 and 5 mM EDTA) with freshly added proteolysis inhibitors. Protein concentrations of total cell lysates were determined by the BCA Protein Assay (Pierce Chemicals Co., Rockford, IL), using bovine serum albumin (BSA) as standard. Total cell lysates were mixed with standard electrophoresis sample buffer. Denatured samples (20 μg proteins) and MW protein markers (Precision Plus Protein™ Standards, Bio-Rad, Hercules, CA) were resolved by 10% SDS-PAGE under reducing conditions. Proteins were subsequently electrotransferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The membrane was then incubated for 3 h at room temperature in a blot solution (10 mM Tris–HCl pH 8, 150 mM NaCl, 0.05% Tween 20 and 7% skim milk) to block nonspecific binding and then cut at the 50 kDa marker band. The upper part of the membrane (containing ER protein) was incubated overnight at 4 °C in a fresh blot solution containing a rabbit anti-human ER-α antibody (G-20) diluted 1:5000, and the lower part (containing the housekeeper actin protein) was incubated in a fresh blot solution containing a mouse anti-actin monoclonal antibody diluted 1:2500 (Chemicon, Temecula, CA). Immunoblots were then incubated for 2 h at room temperature in a blot solution containing peroxidase-labeled donkey anti-rabbit IgG antibody (1:5000) or peroxidase-labeled sheep anti-mouse IgG antibody (1:5000), both obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Bound peroxidase activity was revealed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemicals Co.). The signal associated with immunoreactive material was detected with a LAS-3000 CCD camera (Fujifilm, Düsseldorf, Germany) and the software Image Reader (Raytest®, Straubenhardt, Germany). Immunoreactive band intensity was quantified using the software AIDA® Image Analyser 3.45 (Raytest®).

3. Results

The appearance of control (estrogen-deprived) MCF-7 cells after immunofluorescence staining for ER is illustrated in Fig. 1a. In agreement with previous work of our group (Laïos et al., 2003; Journé et al., 2004), most cells in the field exhibit distinctive fluorescence in their nuclei.

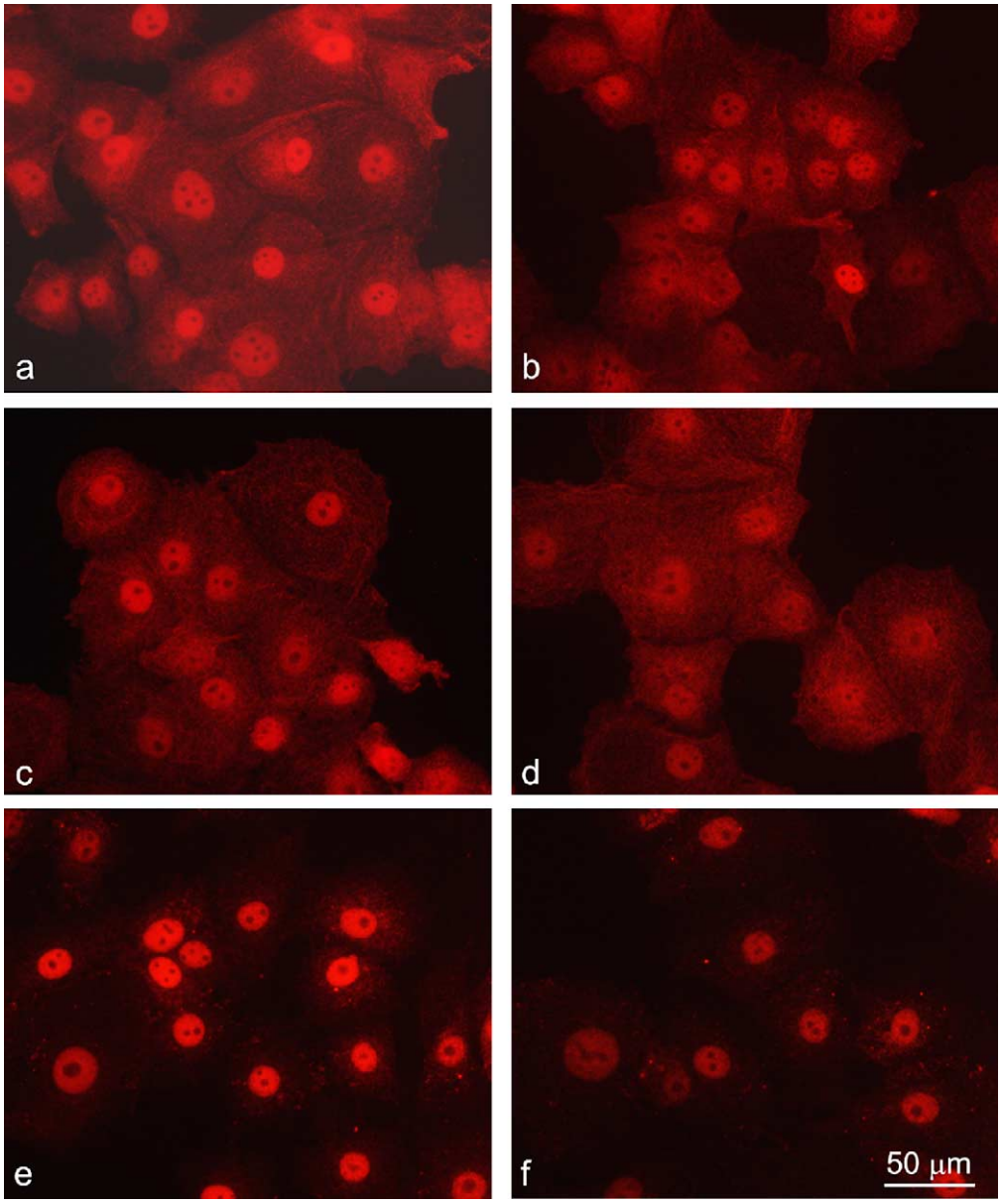

Fig. 1. Effect of radicicol on ER expression in MCF-7 cells, as demonstrated by immunofluorescence staining with HC-20 (a–d) or H-184 antiserum (e, f) after PAF fixation. a, e: untreated cells, b: 2 h-exposure to 10^{-9} M E_2, c: 2 h-exposure to 2×10^{-6} M radicicol, d, f: 6 h-exposure to 2×10^{-6} M radicicol. Texas Red labeling.

Treatment with 2×10^{-6} M radicicol resulted in a progressive decrease of immunofluorescence signal, reflecting the disappearance of immunoreactive ER (Fig. 1c and d). This alteration of immunofluorescence pattern was similar to that associated with E_2-induced ER downregulation (Fig. 1b). Loss of fluorescence was already visible after 2 h of radicicol exposure (Fig. 1c) and appeared virtually complete at 6 h (Fig. 1d). By comparison, the Hsp90 inhibitor geldanamycin at equimolar concentration caused a comparable, but seemingly less marked ER depletion (data not shown). Of note, geldanamycin has a 50-fold lower affinity for Hsp90 as compared to radicicol (Roe et al., 1999). Since no further change in immunofluorescence pattern was noted after 6 h of exposure to radicicol (not shown), this treatment duration was selected for subsequent experiments.

Extinction of ER immunofluorescence signal in MCF-7 cells exposed to radicicol was not related to the use of a particular anti-ER antibody (namely HC-20 directed against F domain of the receptor, Fig. 1a–d) since it could also be observed with other polyclonal antibodies (H-184 directed against A/B domain and G-20 directed against D domain), as well as with monoclonal antibodies (clones B403 and 6F11) (typical data obtained with H-184 antiserum are illustrated Fig. 1e and f). Thus, it could reasonably be assumed that immunofluorescence data illustrated in Fig. 1 and following

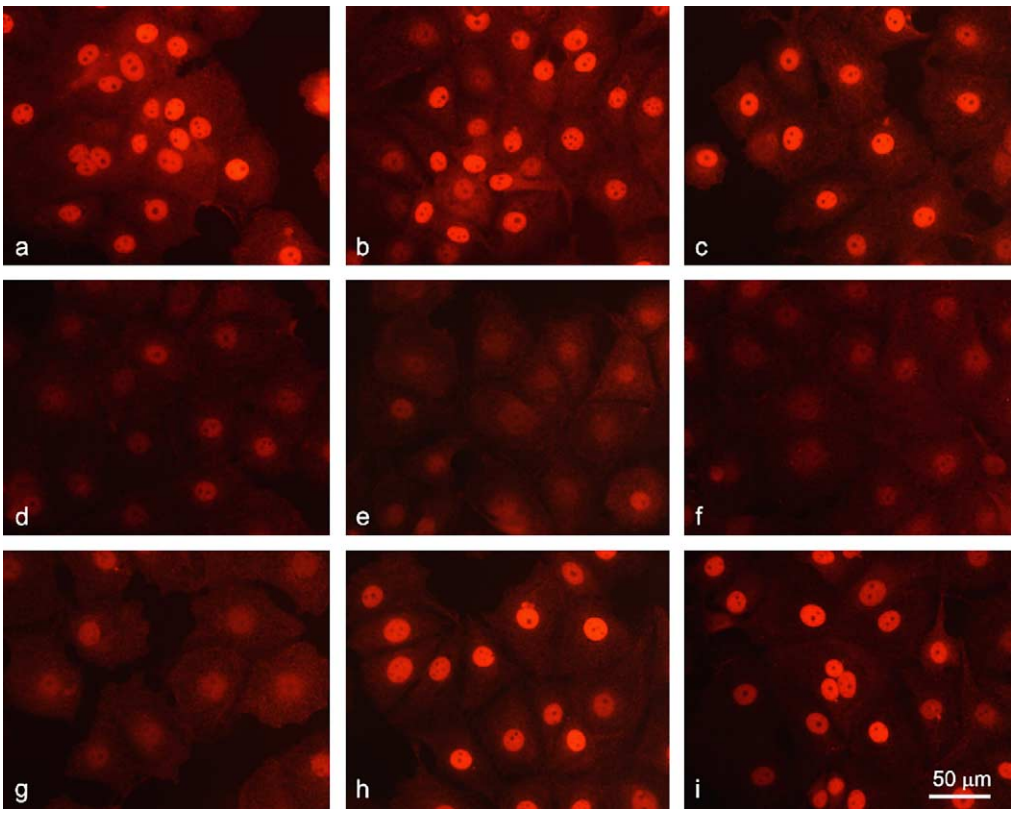

Fig. 2. Modulation of E_2- or radicicol-induced ER depletion in MCF-7 cells by proteasome inhibitors MG-132 and LLnL. a: untreated cells; b: 7 h-exposure to 10^{-5} M MG-132; c: 7 h-exposure to 10^{-4} M LLnL; d–f: cells exposed for 6 h to 2×10^{-6} M radicicol alone (d), in combination with MG-132 (e) or in combination with LLnL (f); g–i: cells exposed for 6 h to 10^{-9} M E_2 alone (g), in combination with MG-132 (h) or in combination with LLnL (i). Treatment with MG-132 or LLnL was initiated 1 h before addition of E_2 or radicicol. ER was demonstrated by immunofluorescence staining with HC-20 antiserum after PAF fixation. Texas Red labeling.

truly reflected ER degradation and not mere change in receptor immunoreactitivity.

It is known that ligand-induced downregulation of ER involves receptor degradation in proteasomes. Therefore, we tested the impact of proteasome inhibitors MG-132 and LLnL on ER decrease resulting from radicicol exposure (Fig. 2). Treatment with MG-132 or LLnL alone barely affected ER immunolabeling even though careful examination revealed a slight increase of immunofluorescence signal (Fig. 2a, b and c). After 6 h of exposure, radicicol and E_2 produced a similar reduction of ER immunoreactivity (Fig. 2d and g). However, MG-132 (Fig. 2h versus g), as well as LLnL (Fig. 2i versus g) in combination with E_2 completely abolished agonist-induced ER downregulation. By contrast, proteasome inhibition had only limited effect on radicicol-induced ER degradation (Fig. 2e and f versus 2d).

It was also important to determine whether observations illustrated in Fig. 2 could be extended to other ER-positive breast carcinoma cell lines. Thus, we examined by immunofluorescence the effect of radicicol with or without MG-132 on ER level in IBEP-2 cells. IBEP-2 cell line has been previously characterized with respect to ER expression and receptor level modulation by estrogen agonists and antagonists (Journé et al., 2004). In particular, these cells exhibit an MG-132-sensitive ER downregulation in response to E_2 (Fig. 3a, e and f). Examination of Fig. 3 reveals that radicicol induces in IBEP-2 cells an MG-132-insensitive ER depletion (Fig. 3a, c and d), similar to that recorded in MCF-7 cells. Similar data were obtained with two different antibodies, namely 6F11 and HC-20. Only observations performed with the latter antibody are shown in Fig. 3.

ER estimation by immunoblotting corroborated findings from immunofluorescence studies (Fig. 4). Thus, cell treatment with radicicol provoked a decline of ER level (approximately 80%) equivalent to that caused by exposure to E_2. E_2-induced ER downregulation was suppressed by MG-132 and LLnL. In contrast, ER depletion caused by radicicol was barely affected by proteasome inhibition.

It is known from previous work (El Khissiin et al., 1997; El Khissiin and Leclercq, 1999) that the protein synthesis inhibitor cycloheximide largely abolishes ER downregulation caused by agonist (E_2) binding. Thus, we investigated the effect of cycloheximide on ER depletion resulting from exposure to radicicol (Fig. 5). As seen before, estrogen-deprived cells in control cultures exhibited a strong nuclear signal associated with ER immunoreactivity (Fig. 5a). Cycloheximide alone did not cause visible change in ER expression within the time frame of the experiment (Fig. 5b). Examination of ER

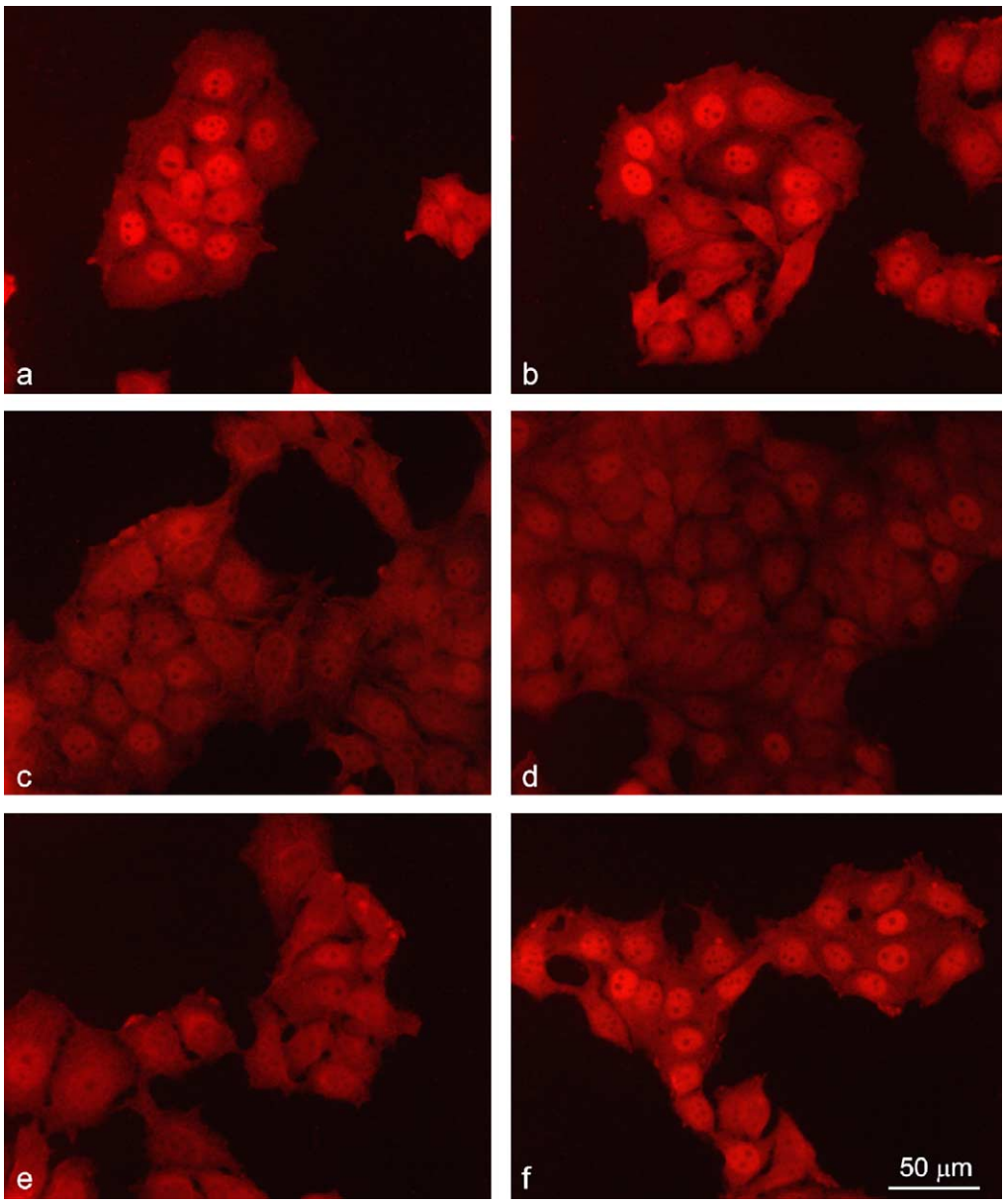

Fig. 3. ER depletion caused by radicicol in the IBEP-2 cell line. a: untreated cells; b: 7 h-exposure to 10^{-5} M MG-132; c, d: 6 h-exposure to 2×10^{-6} M radicicol alone (c) or in combination with MG-132 (d); e, f: 6 h-exposure to 10^{-9} M E_2 alone (e) or in combination with MG-132 (f). MG-132 was added 1 h before radicicol or E_2. ER was demonstrated by immunofluorescence staining with HC-20 antibody after PAF fixation. Texas Red labeling.

immunofluorescence signal in cells treated with E_2 in absence (Fig. 5c) or in presence (Fig. 5d) of cycloheximide confirmed that the latter inhibitor partially suppresses agonist-induced ER downregulation. By contrast addition of cycloheximide in combination with radicicol had no apparent effect on ER depletion produced by radicicol (Fig. 5e and f).

OH-Tam, as well as other triphenylethylene antiestrogens, have been reported to provoke ER upregulation in breast carcinoma cells (Laïos et al., 2003 and references therein, Journé et al., 2004). A possible interference of OH-Tam with radicicol-induced ER degradation was first evaluated in cells pretreated with this antiestrogen. Although it is well known that exposure of MCF-7 cells to OH-Tam results in ER upregulation (Laïos et al., 2003), this upregulation was not associated with a major change of ER immunolabeling in paraformaldehyde (PAF)-fixed cells (compare Fig. 6a and c). However, cells which had accumulated ER protein as a result of OH-Tam treatment proved to be largely refractory to radicicol-induced ER degradation since they exhibited no visible decrease of ER-associated immunofluorescence (Fig. 6a and d), in contrast with cells exposed to radicicol without OH-Tam pretreatment (Fig. 6a and b).

Since PAF fixation does not appear suitable for the morphological demonstration of antiestrogen-induced ER upregulation, an alternative fixation procedure was applied in order to determine whether radicicol would directly interfere

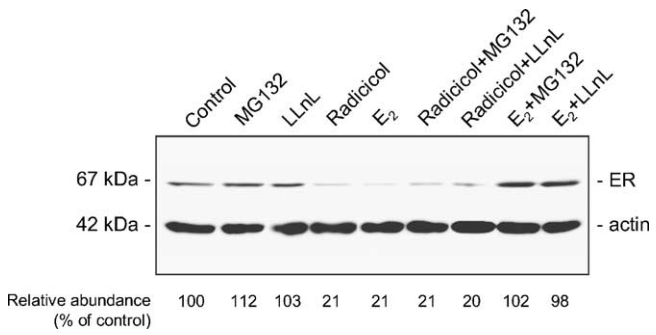

Fig. 4. Effect of radicicol (2×10^{-6} M), E_2 (10^{-9} M), MG-132 (10^{-5} M), LLnL (10^{-4} M) or combinations thereof on steady-state level of ER in MCF-7 cells, as assessed by SDS-PAGE and immunoblotting. Cells were processed for ER analysis after 7 h of treatment with MG-132 or LLnL, or 6 h of treatment with other agents, as specified in Fig. 2. The amount of protein with ER immunoreactivity was evaluated as described in Section 2 and expressed in percentage of control (untreated cells). Actin was used as a loading control.

with the stabilizing effect of OH-Tam on the receptor. When an alcohol-based fixative (Carnoy's mixture) is substituted for PAF, the pattern of immunofluorescence staining with anti-ER antibody undergoes a drastic change consisting in a marked attenuation of the intensity of the nuclear signal (Fig. 6e). Such a modification has been attributed to a more or less extensive extraction of ER protein. Amazingly, in these conditions, cell treatment with OH-Tam provokes an augmentation of ER-associated immunofluorescence (Fig. 6g), this being probably due to drug-induced ER accumulation/anchoring to the nuclear matrix (Oesterreich et al., 2000; Laïos et al., 2003). Strikingly, radicicol added in combination with OH-Tam did not seem to affect ER upregulation induced by the antiestrogen (Fig. 6h). It is noteworthy that, in these experimental conditions, immunofluorescence analysis failed to detect radicicol-induced ER depletion (Fig. 6e and f).

Partial antiestrogens with a benzothiophene structure (e.g. raloxifene, LY 117,018) cause less change in ER expression than triphenylethylene antagonists (Wijayaratne et al., 1999). In particular, immunofluorescence staining after fixation with Carnoy's mixture fails to demonstrate substantial ER signal increase in LY 117,018-treated MCF-7 cells (data not shown). Here, we examined by immunofluorescence staining after PAF fixation the expression of ER in cells exposed to radicicol, LY 117,018 or a combination thereof. Results are illustrated in Fig. 7. As could be expected, no marked change in ER immunofluorescence pattern was seen in cells exposed to LY 117,018 alone (Fig. 7a and c), whereas radicicol produced the typical reduction of ER-associated signal (Fig. 7a and b). Strikingly, combination of LY 177,018 with radicicol abrogated the ER depletion induced by the latter inhibitor (Fig. 7b and d).

Here also, data obtained by ER immunostaining were confirmed by SDS-PAGE and ER immunoblotting (Fig. 8). Thus, cell treatment with OH-Tam for 6 h resulted in a marked ER upregulation (approximately twofold increase in ER expression), whereas exposure to LY 117,018 caused less change in ER steady-state level. Regardless of their effect on ER turnover, both antiestrogens stabilized the receptor and prevented its degradation in radicicol-treated cells. On the other hand, E_2 diminished, rather than enhanced, ER loss consecutive to radicicol exposure, suggesting that ER stability was no longer dependent on Hsp90.

4. Discussion

The capacity of target cells to react to steroid stimulation is determined by the intracellular level of cognate receptors (Vanderbilt et al., 1987; Webb et al., 1992, Alarid et al., 1999). Intracellular ER concentration is influenced both by receptor synthesis (depending on gene transcription and mRNA stability) and breakdown. ER turnover occurs quite rapidly since the estimated half-life for the unliganded receptor is approximately, 4–5 h (Wijayaratne and McDonnell, 2001 and references therein). As shown by studies relying on the use of specific inhibitors (MG-132, lactacystin, LLnL), ER degradation mostly occurs in proteasomes (Alarid et al., 1999; El Khissiin and Leclercq, 1999; Nawaz et al., 1999). Recent studies also suggest that ER ubiquitination and/or proteasomal degradation might be pivotal for receptor-mediated gene transactivation (Lonard et al., 2000, Lonard and Smith, 2002; Nawaz and O'Malley, 2004).

Unliganded, native ER forms molecular complexes with chaperones and cochaperones. Actually, receptor-chaperone complexes behave like dynamic, evolving entities since they progress through a cycle characterized by the assembly/deassembly of different chaperones and cochaperones (Richter and Buchner, 2001). According to current models, neosynthesized ER first interact with a chaperone/cochaperone complex ("foldosome") consisting of Hsp90, HOP, Hsp70 and Hsp40. Then, receptor "activation" (i.e. post-translational folding) proceeds through the substitution of p23 and immunophilins for HOP, Hsp70 and Hsp40 (Pratt and Toft, 2003). Hsp90 definitely plays a major role in the progression of receptor-chaperone complex through the activation cycle. This chaperone is an adenine nucleotide-binding protein which becomes active once in the ATP-bound form. Radicicol and geldanamycin compete with ADP/ATP for the nucleotide binding pocket and thereby disrupt Hsp90 activity (Scheibel and Buchner, 1998; Piper, 2001, Maloney and Workman, 2002).

Molecular chaperones ensure correct folding of neosynthesized ER, prime it for ligand binding (Fliss et al., 2000) and prevent gene transactivation by unactivated receptor. Most importantly, chaperones, such as Hsp90 also protect unliganded receptor from unwanted degradation (Bagatell et al., 2001). The current observations based on ER immunofluorescence and immunoblotting show unequivocally that Hsp90 inhibition by radicicol leads to a rapid loss of ER in MCF-7 and IBEP-2 cells.

As it is the case for other steroid receptors, agonist binding to ER results in a rapid decrease of receptor level (downregulation) (El Khissiin and Leclercq, 1999, Nawaz, et al., 1999;

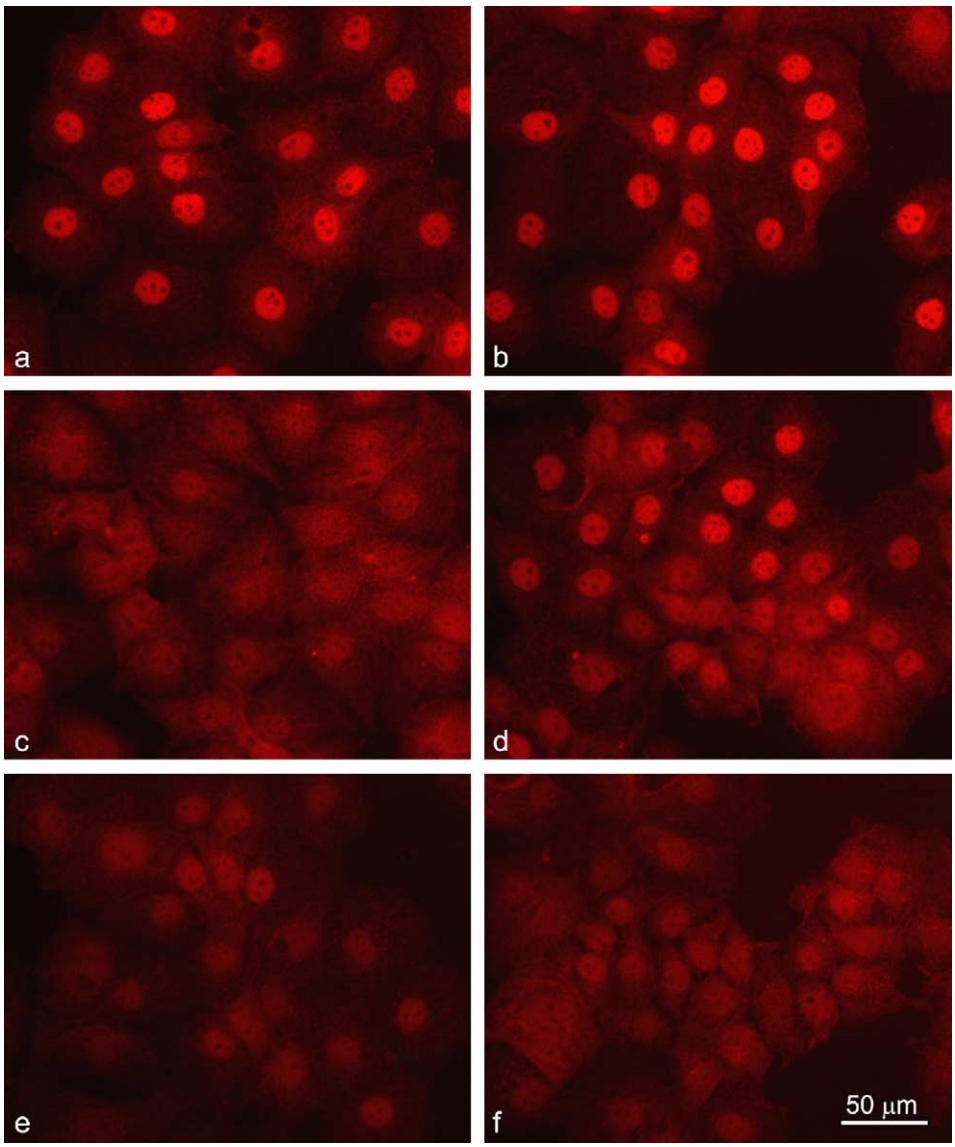

Fig. 5. Influence of the protein synthesis inhibitor cycloheximide on E_2- or radicicol-induced ER depletion in MCF-7 cells. a: untreated cells; b: 7 h-exposure to 5×10^{-5} M cycloheximide; c, d: cells exposed for 6 h to 10^{-9} M E_2 alone (c) or in combination with cycloheximide (d); e, f: cells exposed for 6 h to 2×10^{-6} M radicicol alone (e) or in combination with cycloheximide (f). Treatment with cycloheximide was initiated 1 h before addition of E_2 or radicicol. ER was demonstrated by immunofluorescence staining with HC-20 antiserum after PAF fixation. Texas Red labeling.

Wijayaratne and McDonnell, 2001). In the short term (i.e. within hours following hormone exposure), the decline of ER protein concentration is mostly due to an enhancement of ER breakdown (Dauvois et al., 1992; Alarid et al., 1999; Alarid et al., 2003). Although this has not been formally established, it is widely assumed that agonist-induced ER downregulation contributes to prevent cell overreaction to estrogenic stimulation. Amazingly enough, this safeguard mechanism persists in cell lines derived from breast carcinoma (MCF-7, IBEP-2) (Journé et al., 2004), despite cell dysregulation associated with neoplastic transformation.

In this study, immunofluorescence staining as well as immunoblot analysis reveal that ER degradation subsequent to radicicol exposure is barely affected by proteasome inhibitors MG-132 and LLnL. It is noteworthy that, in similar experimental circumstances, ER downregulation due to E_2 binding is totally reversed by both inhibitors. Thus, under circumstances where proteasome blockage abrogates physiological ER downregulation (i.e. caused by a natural agonist), it fails to prevent ER degradation caused by chaperone inhibition. It must be noted here that LLnL (also called calpain inhibitor I) differs from MG-132 in as much as it has a larger inhibitory spectrum (Kisselev and Goldberg, 2001). At first sight, one might be tempted to assume that ER destabilized by radicicol is degraded by proteolytic enzymes extrinsic to the ubiquitin-proteasome system. Yet, such an interpretation is not in accordance with the currently held concept of a close association between chaperones and proteasomes (Connell et al., 2001; Jiang et al., 2001). Alternatively, the incapacity of MG-132 and LLnL to prevent radicicol-induced ER degrada-

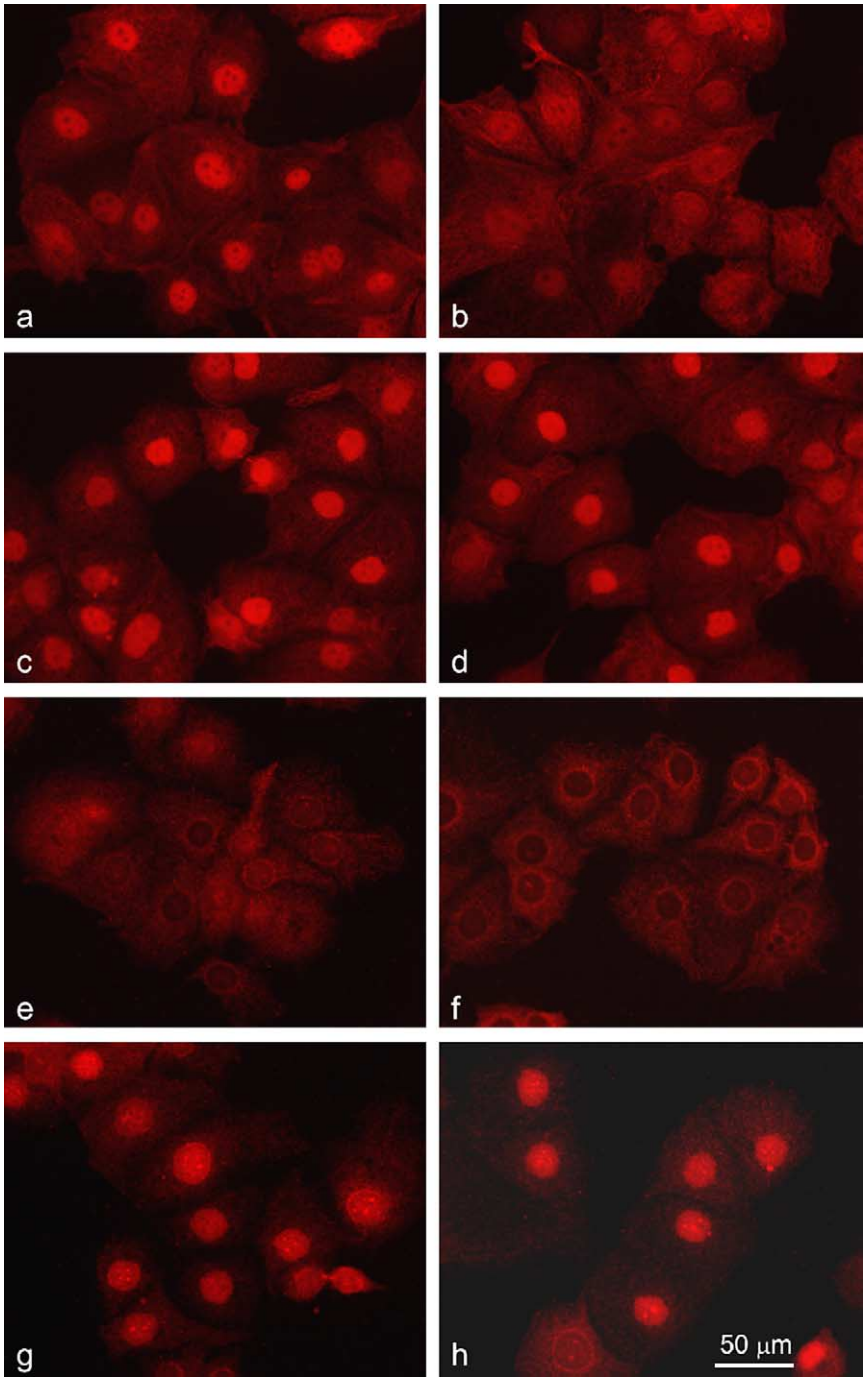

Fig. 6. Suppression of radicicol-induced ER depletion by OH-Tam in MCF-7 cells. ER immunofluorescence staining with HC-20 antiserum after culture fixation with PAF (a–d) or Carnoy's mixture (e–h). a, e: untreated cells; b, f: 6 h-exposure to 2×10^{-6} M radicicol; c: cells exposed for 24 h to 10^{-7} M OH-Tam (cultures fixed 6 h after drug withdrawal); d: 24 h-exposure to OH-Tam followed by 6 h-exposure to radicicol; g, h: 6 h-exposure to OH-Tam alone (g) or in combination with radicicol (h). Texas Red labeling.

tion could stem from the fact that proteasomes contain several proteolytic activities and exhibit broad specificity (Bogyo and Wang, 2002). Following this line of reasoning, one could surmise that agonist- and radicicol-induced ER degradation, even though it probably occurs in the proteasome compartment, involves separate enzymatic activities differing with respect to inhibitor sensitivity. Another possibility would be that ER destabilized by chaperone inhibition is destroyed in a proteasome subpopulation less accessible to some inhibitors. In this context, previous work by Lee et al. (2002) reports that cell treatment with LLnL for a period of 24 h (instead of 7 h as it is the case in our experiments) efficiently blocks ER degradation in cells exposed to radicicol. However, it should be kept in mind that a 24 h period of proteasome inhibition can lead per se to a substantial ER accumulation resulting from drug-induced al-

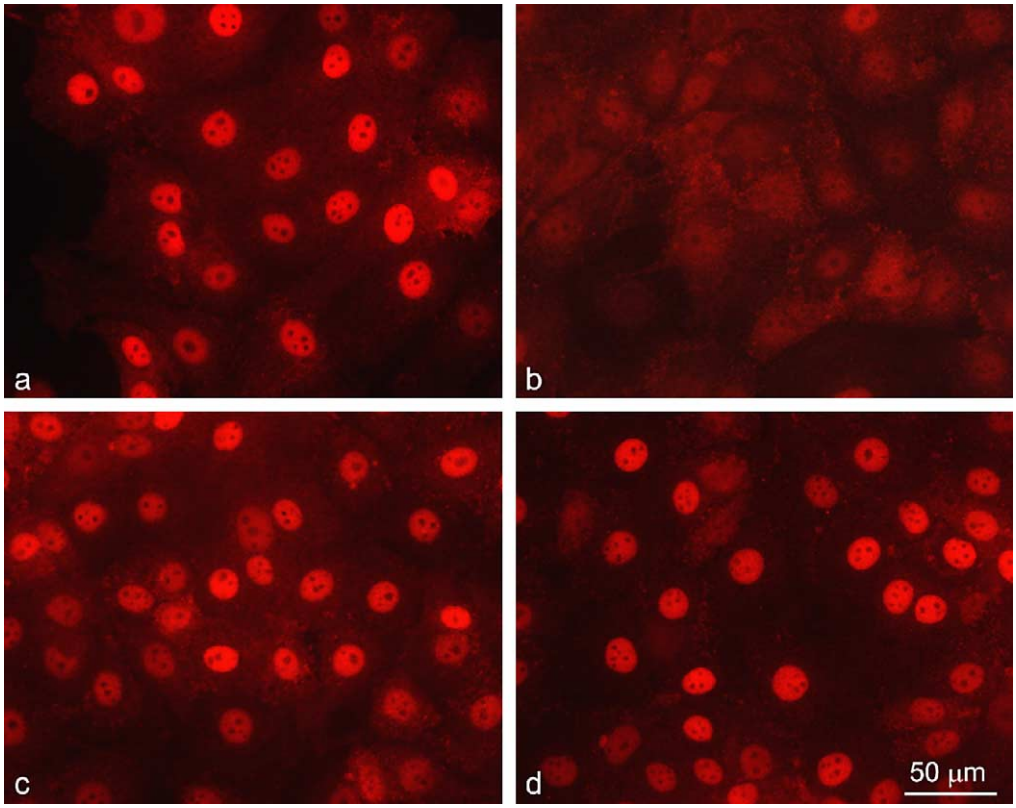

Fig. 7. Suppression of radicicol-induced ER depletion by LY 117,018 in MCF-7 cells. a: untreated cells; b, d: cells treated for 6 h with 2×10^{-6} M radicicol alone (b) or in combination with 10^{-7} M LY 117,018 (d); c: cells treated for 6 h with LY 117,018 alone. ER was demonstrated by immunofluorescence staining with HC-20 antiserum after PAF fixation. Texas Red labeling.

teration of ER basal turnover (Laïos et al., manuscript in preparation).

Incidentally, findings similar to those reported here were made in a recent study on the regulation of the androgen receptor (AR). Like ER, AR is stabilized by interactions with molecular chaperones. Chaperone-mediated folding of client proteins is inhibited by a particular cochaperone, C-terminal

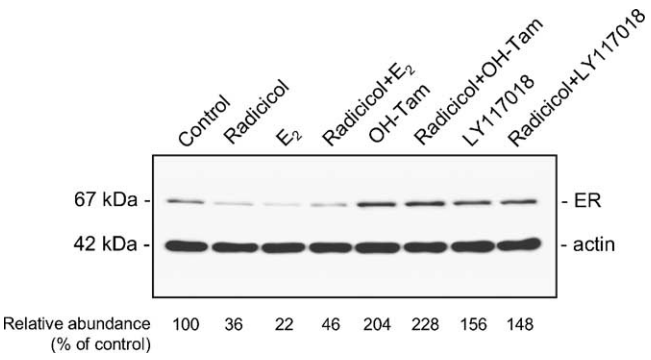

Fig. 8. Effect of radicicol (2×10^{-6} M), E_2 (10^{-9} M), OH-Tam (10^{-7} M), LY 117,018 (10^{-7} M) or combinations thereof on steady-state level of ER in MCF-7 cells (SDS-PAGE and immunoblotting). Cells were processed for ER analysis after 6 h of drug exposure, as indicated in legend to Fig. 4. Actin was used as a loading control.

Hsp-interacting protein (CHIP), which also functions as a E3 ubiquitin ligase (Murata et al., 2003). As recently reported by Cardozo et al., CHIP overexpression results in a drastic decline in AR steady-state level. In this situation which resembles that created by radicicol-induced Hsp90 inhibition, the authors observe that AR decline is not completely reversed by proteasome inhibitors (Cardozo et al., 2003).

In another perspective, the possibility of variation in the pathway underlying ER breakdown has also been suggested by previous work showing that ER downregulation consecutive to E_2 binding is abrogated by protein synthesis inhibition (cycloheximide), whereas receptor downregulation caused by pure antiestrogens is not (El Khissiin and Leclercq, 1999). This intriguing observation has been confirmed recently by Marsaud et al. (2003) who concluded from their findings that more than one regulatory pathway could commit ER to degradation. Incidentally, we found in this study that radicicol-induced ER degradation is also insensitive to cycloheximide. In view of the current results, it would be worth exploring further what differentiates in mechanistic terms the degradation of unliganded ER, resulting from Hsp90 inhibition, from the physiological downregulation of agonist-bound receptor.

Unlike estrogen agonists and pure antagonists which cause ER downregulation, some (but not all) partial antiestrogens provoke receptor upregulation. In particular, previous work of

our group reveals that the triphenylethylene antagonist OH-Tam stabilizes ER and prevents its breakdown, and thus leads to receptor accumulation (Laïos et al., 2003). Data reported herein indicate that ER accumulated upon cell exposure to OH-Tam becomes refractory to radicicol-induced degradation, even when the antiestrogen is withdrawn. This is consistent with the fact that ER accumulation caused by OH-Tam persists for at least 24 h after suppression of the antiestrogen, even though the process of ER upregulation is not irreversible (i.e. receptor accumulation rapidly vanishes when OH-Tam-treated cells are subsequently exposed to E_2 or a pure antiestrogen) (Laïos et al., 2003). As can be inferred from the current observations, ER interacts with OH-Tam in such a way that it does not reacquire the ability to associate with Hsp90. This distinguishes OH-Tam from estrogen agonists since it has been shown that E_2-ER interactions do not preclude subsequent Hsp90 binding to the receptor, at least in vitro (Bouhouche-Chatelier et al., 2001).

The current study, as well as previous work (Laïos et al., 2003), reveals that OH-Tam-induced ER upregulation results in a conspicuous increase of ER immunofluorescence signal in cells fixed with Carnoy. This phenomenon is consistent with the antiestrogen-mediated anchoring of ER to the nuclear matrix (Giamarchi et al., 2002; Maruvada et al., 2003) via the recruitment of corepressors (Oesterreich et al., 2000). Although the actual nature of the noncovalent bonds responsible for this anchoring remains unknown, they seem to entail neither hydrophobic (OH-Tam-bound ER appears unextractable by organic solvents), nor electrostatic interactions (OH-Tam-receptor complexes maintain some solubility in low salt cell extracts) (Marsaud et al., 2003). Whatever its mechanism may be, increase of ER immunofluorescence signal due to OH-Tam is not modified by radicicol. Similarly, OH-Tam-induced ER accumulation demonstrated by immunoblotting appears insensitive to radicicol, indicating that the latter inhibitor is unable to impede ER upregulation. The most likely explanation for this finding is that Hsp90 is displaced from the receptor upon OH-Tam binding, in accordance with what is known of chaperone interactions with nuclear receptors (see Pratt, 1997b, for review). One must note that ER demonstrated by immunofluorescence staining in OH-Tam-treated cells exhibits a nuclear localization, regardless of treatment with radicicol. Inasmuch as Hsp90 is essentially a cytoplasmic protein (Passinen et al., 2001), it probably does not colocalize with ER in the nuclear compartment.

Altogether, our data imply that OH-Tam-bound ER becomes resistant to Hsp90 inhibition because it is no longer associated with chaperones. Thence, it ensues that ligand binding to ER could be a more critical factor than receptor upregulation in the resistance to radicicol-induced destabilization. Data obtained with LY 117,018 appear in agreement with this view. This compound is an analog of raloxifene (Schafer et al., 2001), and thus belongs to the benzothiophene group of partial antiestrogens. Benzothiophene antagonists cause less ER accumulation than triphenylethylene derivatives in breast carcinoma cells (Wijayaratne et al., 1999). Besides, at variance with steroid antiestrogens, benzothiophenes have no tendency to provoke ER downregulation. Indeed, in the case of these compounds, the balance between degradation and synthesis of ER is more in favor of degradation than in the case of triphenylethylene antagonists (Laïos et al., 2003). Nevertheless, it is clear from the data herein that LY 177,018, like OH-Tam, abolishes radicicol-induced ER degradation.

As shown by our data and recent work by others (see below), ER binding by partial antiestrogens stabilizes the receptor, making it immune to degradation induced by Hsp90 inhibition. In this setting, partial antiestrogens should only protect ER and not other Hsp90 client proteins. A recent report by Beliakoff et al. (2003) reveals that indeed such is the case. In MCF-7 cells and tamoxifen-resistant MCF-7 variants exposed to geldanamycin, tamoxifen prevents the disappearance of ER but not that of Akt and Raf-1 which are other Hsp90 client proteins. Furthermore, the same study shows that tamoxifen does not modify the effect of geldanamycin on the progression of ER-chaperone complexes (i.e. inhibition of p23 recruitment by these complexes). Actually, there is no discrepancy between this finding and the fact that tamoxifen stabilizes ER by binding the receptor and dissociating it from chaperones. Indeed, tamoxifen and geldanamycin could have the same inhibitory effect on p23-ER association, the former by disrupting Hsp90 activity (inhibition of p23 recruitment), the latter by acting as a ligand and inducing the dissociation of ER-chaperone complexes.

Consistently with the stabilizing action of partial antiestrogens on ER, we also noted that E_2 and radicicol did not produce ER degradation in an additive fashion. The most likely explanation to this finding is that agonist-bound ER undergoes downregulation without being really affected by Hsp90 inhibition. Although such an interpretation needs further confirmation, it reinforces the concept that ligand-independent and agonist-induced ER degradation might somehow occur through distinct pathways.

To conclude, the current study discloses the possible existence of different enzymatic activities and/or degradative pathways responsible for ER breakdown in breast carcinoma cells. In addition, our findings reveal that ER stabilized by partial antiestrogens becomes resistant to degradation subsequent to Hsp90 inhibition.

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