Influence of oestradiol and tamoxifen on oestrogen receptors-α and -β protein degradation and non-genomic signalling pathways in uterine and breast carcinoma cells

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Abstract

Tamoxifen acts as an oestrogen antagonist in the breast reducing cell proliferation, but in the uterus as an oestrogen agonist resulting in increased cell proliferation. Tamoxifen exerts its tissue-specific effects through the oestrogen receptors (ERα or ERβ). The levels and functions of the two ERs affect the response of the target tissue to oestrogen and tamoxifen. We examined the control of ER stability in breast and uterine cell lines using western blotting and RT-PCR. In MCF-7 breast-derived cells, ERα and ERβ proteins were rapidly degraded via the proteasome pathway in response to oestradiol; conversely tamoxifen stabilised both receptors. In Ishikawa uterine-derived cells, oestradiol and tamoxifen stabilised ERα but led to degradation of ERβ by the proteasome pathway. Further investigations showed that oestradiol induced activation of the non-genomic ERα/Akt signalling pathway in MCF-7 cells. We have demonstrated that the alternative Erk signalling pathway is activated in Ishikawa cells following oestradiol treatment in the absence of an active proteasome pathway and therefore increased levels of ERβ. In conclusion, our data have demonstrated tamoxifen or oestradiol control of ER subtype stability and that non-genomic activation of transcription pathways is cell specific.

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Introduction

Tamoxifen is widely used for the adjuvant treatment of breast cancer, although epidemiological evidence has shown an increase in endometrial cancers in treated women (Rutqvist *et al.* 1995, Fotiou *et al.* 2000, Kloos *et al.* 2002). In the breast, tamoxifen acts as an oestrogen antagonist, reducing or preventing the proliferation of oestrogen receptor (ER)-positive tumour cells (Cuzick *et al.* 2003, Power & Thompson 2003). However, in the uterus, tamoxifen acts as an oestrogen agonist resulting in cell proliferation (Goldstein 2001, Pole *et al.* 2005).

Tamoxifen and other selective ER modulators (SERMs) exert their tissue-specific effects through interaction with one or both of the ERs (ERα or ERβ) (McDonnell *et al.* 2002, Park & Jordan 2002). In the classical pathway for ER activation, ligand binding causes the receptors to undergo conformational changes and dimerise forming homo- or heterodimers, which bind to the palindromic oestrogen response element (ERE), leading to recruitment of coactivator proteins and transcription of oestrogen responsive genes (Edwards 2000, Klinge 2001). Recent studies have reported the importance of cell membrane-associated ER (Kelly & Levin 2001) in the activation of cytoplasmic signalling cascades, although there are considerably fewer membrane than nuclear receptors (Razandi *et al.* 1999). Rapid non-genomic signalling through pathways involving phosphotidylinositol-3 kinase (PI3K)/Akt or Erk1/Erk2, leading to the transcription of target genes, has been described (Pedram *et al.* 2002, Song *et al.* 2002), suggesting that some of the proliferative effects of oestradiol may be mediated through these mitogenic pathways. This non-genomic effect of oestrogens has added to the broad range of transcriptional responses that can be produced by ligand-bound ER in different cell types.

Control of ERα protein levels has been studied within various cell types and it is now generally accepted that this protein is targeted for rapid degradation via the ubiquitin–proteasome pathway in response to oestradiol in breast cancer cells (Dowsett & Ashworth 2003). The control and degradation pathways for ERβ remain unclear and as yet there is not a universally accepted

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mechanism for the control of levels of either ERα or ERβ in uterine cells. ERα and ERβ proteins may be degraded differentially in breast and uterine cells in response to oestradiol and tamoxifen, thus providing some mechanistic evidence for the diverse response of these tissues. There is evidence to suggest that the activity of the proteasome pathway controlling ERα degradation in MCF-7 cells is directly linked to activation of transcription through the ERE (Reid *et al.* 2003).

If the magnitude of transcriptional activity within cells is directly related to the cellular concentration of the ER, regulatory mechanisms controlling ER protein levels could alter its transcriptional output. In this paper we explore the activation of alternative mitogenic non-genomic signalling pathways. Further aims of this study were to establish previously undefined mechanisms for ERβ degradation in the MCF-7 cell type. Comparisons were made with ERα and ERβ protein degradation in the uterine adenocarcinoma-derived Ishikawa cell line. The differential effect of oestradiol or tamoxifen on ER protein levels and subsequent activation of the non-genomic signalling pathways was investigated.

Materials and methods

Human cell culture

Human breast adenocarcinoma-derived MCF-7 cells (ECACC, Salisbury, Wiltshire, UK; were maintained in Dulbecco's modified Eagles' media (DMEM)/F12 (Invitrogen) supplemented with 2 mM glutamax (Invitrogen) and 10% foetal calf serum (FCS; Invitrogen). Human uterine epithelial carcinoma-derived Ishikawa cells (ECACC) were maintained in RPMI-1640 media (Invitrogen) supplemented as described above. For visualisation of ERβ by western blotting, Ishikawa cells were transfected with an ERβ expression construct as described previously (Jones *et al.*1999) prior to treatment. All media were phenol red free and cell cultures were maintained in media containing 10% dextran-coated charcoal-stripped (DCST) FCS for 72 h before dosing, to ensure depletion of hormones and growth factors in the calf serum.

Western blotting and protein analysis

Cells were dosed with culture media containing the peptide aldehyde proteasome inhibitor MG115 (10^{-6} M) (Sigma) and incubated for 1 h at 37 °C, 17β-oestradiol (E_2; Sigma) or 4-hydroxytamoxifen (TAM; Sigma) were added at 10^{-8} M and 10^{-6} M respectively for 3 h at 37 °C. For selective inhibition of PI3K/Akt and MEK/Erk1/2 signalling pathways the MCF-7 cells were treated with 50μM LY294002 for 60 min prior to dosing with E_2 (inhibition of PI3K phosphorylation of Akt) and the Ishikawa cells were treated with 30μM PD98059 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) in combination with E_2 for inhibition of MEK phosphorylation of Erk1/2. Cells were washed with phosphate-buffered saline (PBS) and lysed in ice-cold protein extraction buffer (Song *et al.* 2002). For preliminary analysis of ER cellular localisation, nuclear and cytosolic fractions were produced using cells grown in culture media supplemented as above for 72 h and prepared with the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce, Perbio Science, Northumberland, UK) according to the manufacturer's instructions. Protein concentration was determined using the BCA protein assay kit (Sigma) and measured using a BMG fluorostar plate reader (BMG Lab Technologies, Offenburg, Germany) at 540 nm. Proteins were analysed by PAGE (7·5% gels) and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Amersham, Bucks, UK). Positive controls for antibody specificity were 10 ng recombinant protein (rhERα and rhERβ; Calbiochem, Nottingham, UK). Membranes were blocked in 5% (w/v) non-fat milk in 0·1% PBS Tween-20 for 12–18 h. ERα protein was detected with H-184 rabbit polyclonal antibody (Santa Cruz Biotechnologies) and ERβ with 06–629 rabbit polyclonal antibody (Upstate Biotechnologies, Dundee, UK) or GR-39 mouse monoclonal (Oncogene). Akt protein was detected with SC-5289 mouse monoclonal (Santa Cruz Biotechnologies) and phosphorylated Akt (pAKT) with 92715 rabbit polyclonal (Cell Signaling, Hertfordshire, UK). Western blots were re-probed with antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (Biogenesis Ltd, Poole, UK). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnologies) and the Pierce super signal chemiluminescence detection system were used to visualise immunoreactivity. Data were collected using a Syngene (Cambridge, Cambs, UK) GeneGnome gel documentation system and protein expression was normalised to GAPDH levels. Statistical analysis of variance (ANOVA) or two sample *t*-tests were performed where appropriate ($P \leq 0{\cdot}05$).

Fluorescence-based determination of proteasome activity using Suc-LLVY-AMC

Proteasome activity was assessed by a peptide substrate succinyl-LLVY-7-amino-4-methylcoumarin (Suc-LLVY-AMC) for the proteasome complex. Protein extract (100 μM) was incubated with 50μM Suc-LLVY-AMC (Bachem Biochemica, St. Helens, UK) in a total volume of 200μl with 5 mM $MgCl_2$, 5 mM ATP, 50 mM Tris–HCl, pH 7·8, 20 mM KCl and 5 mM magnesium acetate for 1 h at 37 °C, terminated by 200μl 0·1 M sodium borate. The fluorescence of aminomethyl-coumarin was measured in a fluorometer. Standard

curves were prepared containing 7-amino-4-methylcoumarin using the same reagent buffers.

Confocal microscopy

MCF-7 or Ishikawa cells were grown for 72 h in four-well glass chamber slides in culture medium supplemented with charcoal-stripped serum as detailed above. Cells were pretreated with MG115 (10^{-6} M) for 1 h before dosing with E_2 (10^{-8} M), TAM (10^{-6} M) or ethanol vehicle (control) for 3 h. Cells were fixed for 30 min in methanol at −20 °C, washed in PBS and permeabilised with 0·4% Triton X-100 10 min before blocking with 2% normal goat serum/4% BSA for 30 min at 37 °C. Primary mouse monoclonal antibodies were incubated at 4 °C overnight. ERα was detected with Santa Cruz Biotechnologies D12 at 1:20 dilution in blocking buffer and ERβ with Serotec (Oxford, UK) MCA1973S at 1:20 dilution also in blocking buffer. Secondary antibody was AlexaFluor 488 (Molecular Probes, Eugene, OR, USA) at 1:500 in blocking buffer, incubated at 4 °C overnight in darkness. Hoechst 33342 dye was subsequently added for nuclear staining. Confocal microscopic images were captured using a Zeiss LSM 510 META multi-photon confocal microscope (Carl Zeiss (UK) Ltd, Welwyn Garden City, Herts, UK). Low magnification Z-series were collected with a 20 × Plan-neofluar lens and high resolution series using a 40 × 1·3 oil immersion Plan-apocromat lens or a 63 × C-apochromat 1·2 na water immersion lens. Three-dimensional reconstructions of each data set were performed to examine each confocal Z-series using Zeiss Advanced Imaging Microscopy (Carl Zeiss Ltd). Nuclear staining was performed using Hoechst 33258 (10μg/ml; Molecular Probes) in PBS for 10 min and excited in multi photon mode with a Tsuanmi Infra red laser (Spectra Physics, Mountain View, CA, USA) at 760 nm and detected with a 365–405 nm band pass filter. AlexaFluor 488-labelled secondary antibody (Molecular Probes) was excited with a 488 nm Argon laser line (LASOS Lastertechnik GMBH, Germany) and detected using a long pass 505 nm filter.

mRNA extraction and RT-PCR

MCF-7 and Ishikawa cells were counted before lysis and mRNA extraction by oligo dT-linked Dynabeads (Dynal, Biotech Ltd, Wirral, UK) according to the manufacturer's instruction. Dynabead-linked mRNA was used as a template for reverse transcription at 42 °C for 1 h (Promega reagents). Hot-start PCR performed with GAPDH was a control (Hall *et al.* 1998). PCR primer sequences for ERα and ERβ have been described previously (Tschugguel *et al.* 2003). Data were collected using a Syngene GeneGnome gel documentation system and expression was normalised to GAPDH levels.

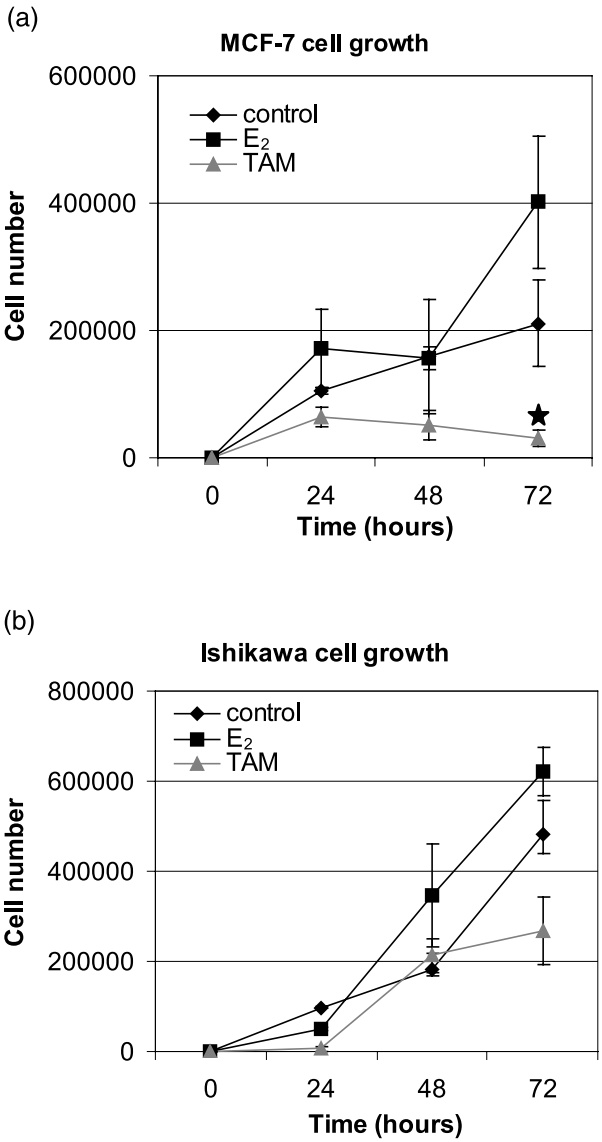

Figure 1 Representative growth curves for (a) MCF-7 and (b) Ishikawa cells and the effects of oestradiol or tamoxifen. Cells maintained in phenol red-free DMEM/F12 or RPMI containing 10% DCST-FCS for 72 h before dosing with 10^{-8} M E_2, 10^{-6} M TAM or vehicle (ethanol control). Cells were counted at 24, 48 and 72 h after dosing using a haemocytometer. Data are corrected for variation in cell number at time 0 and formulated from mean values±S.E. of three experiments. *$P \leq 0{\cdot}05$ (ANOVA).

Results

Oestradiol-induced degradation of ERα is cell type specific

The effect of tamoxifen on proliferation of the breast and uterine cell types was investigated. E_2 stimulated cell proliferation in both cell types (Fig. 1). TAM (10^{-6} M) significantly ($P \leq 0{\cdot}05$) inhibited cell proliferation in the

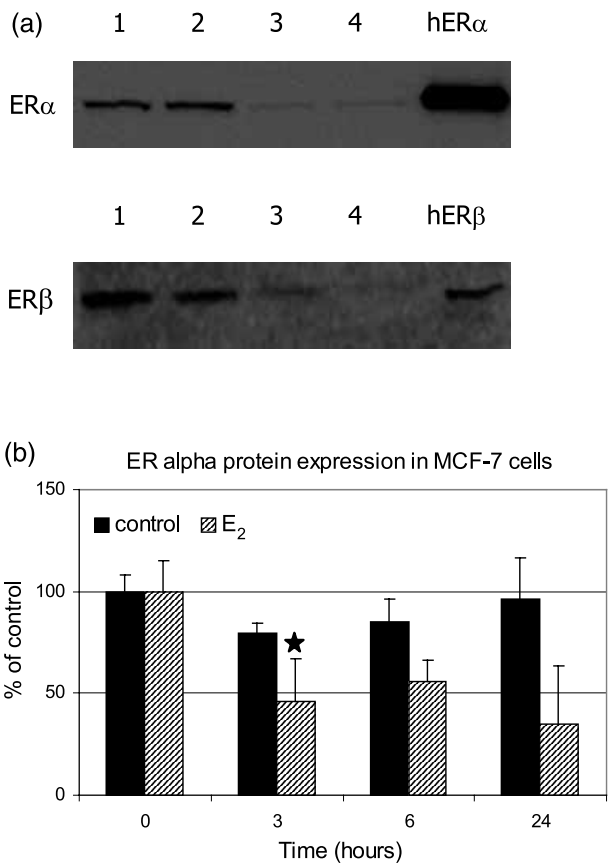

Figure 2 Detection of ER proteins within whole cell lysates. (a) Whole cell lysates (50μg) of MCF-7 (lanes 1–2), Ishikawa (lanes 3–4) and 10 ng of appropriate recombinant protein separated using PAGE western blots, probed with Santa Cruz 184 (human ERα) or Oncogene GR-39 (human ERβ) primary antibodies. (b) ERα protein expression from Western blots of 50μg MCF-7 whole cell lysates maintained in phenol red-free DMEM/F12 containing 10% DCST-FCS for 72 h before dosing with 10^{-8} M E_2. Cells were lysed at 0, 3, 6 and 24 h. Expression levels are expressed as percentage of ERα protein at time 0. Results represent the mean±S.E. of three experiments. *$P \leq 0{\cdot}05$ (ANOVA).

MCF-7 cell line (Fig. 1a). This anti-proliferative effect was not observed to the same degree in the Ishikawa cell line, indicating a partial oestrogen agonist effect in these cells (Fig. 1b). Antibody specificity is shown in Fig. 2a, where recombinant human ERα and ERβ proteins were detected, no other non-specific binding of the antibodies was observed and only a band of the correct molecular weight for the wild-type protein forms was visualised from the cell lysates. A representative time-point at which to study the effect of E_2 treatment on ERα protein was determined in MCF-7 cells. ERα protein diminished over a 24-h period in MCF-7 cells in a time-dependent manner. The earliest significant decrease was observed at 3 h where treatment with E_2 resulted in an $\sim$50% ($P \leq 0{\cdot}05$) loss of ERα (Fig. 2b).

The peptide aldehyde proteasome inhibitor MG115 has previously been demonstrated to have a role in apoptosis (Lopes *et al.* 1997). The toxicity of MG115 was therefore assessed by a trypan blue exclusion assay in MCF-7 and Ishikawa cells. The viability of both cell types remained unaffected after 1-h pretreatment with MG115, and lysis after 3-h incubation with E_2 or TAM (Fig. 3a and b). To assess the efficacy of proteasome pathway inhibitors, the effects of MG115, MG132 or lactacystin on proteasome activity in MCF-7 cells were determined by a fluorescence assay based on the hydrolysis of a peptide substrate (Suc-LLVY-AMC) for the proteasome complex (Nandi *et al.* 1997, Kisselev *et al.* 1999). Results showed that MG115 is as efficient as others, alone or in combination (Fig. 3c). To address the observation that the peptide was still subject to degradation even in the presence of the proteasome inhibitors, the cells were treated with a cocktail of protease inhibitors, in combination with the MG115 proteasome inhibitor. In this additional experiment, full inhibition was achieved suggesting that this peptide is also a target for the protease pathway.

The effect of E_2 or TAM on ERα protein levels was assessed in MCF-7 and Ishikawa cells. ERα protein levels were reduced in MCF-7 cells dosed for 3 h with E_2 (Fig. 4a). Pretreatment with the proteasome inhibitor MG115 for 1 h before dosing with E_2 abolished this effect and led to an increase of protein above control level. It is interesting to note that MG115 treatment alone led to significant stabilisation of the ERα protein ($P \leq 0{\cdot}05$; Fig. 4a). As ERα contains a PEST (rich in proline, glutamate and aspartate, serine and threonine) sequence (Pakdel *et al.* 1993) which is recognised by proteases including calpains, we pretreated the cells with 2S,3S-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester E-64-d (calpain inhibitor) in place of MG115. In this experiment, E_2-induced degradation of ERα was still observed, eliminating the calpain protease as a possible degradation pathway (data not shown). Ishikawa cell lysates did not show any reduction in ERα levels following E_2 treatment compared with control (Fig. 4b) and treatment of the cells with MG115 alone did not lead to a significant increase in ERα protein levels. Western blot analysis of MCF-7 and Ishikawa cell lysates dosed for 3 h with TAM did not show a decrease of ERα protein in either case (Fig. 4a and b).

Tamoxifen-induced degradation of ERβ is also cell type specific

MCF-7 cells, after 3 h exposure to E_2, showed significant rapid degradation of ERβ protein ($P \leq 0{\cdot}05$; Fig. 4c). When cells were pretreated with MG115, levels of ERβ remained similar to those of control cells. In the Ishikawa cells, reduction in ERβ protein was also observed in response to E_2 treatment; pretreatment of

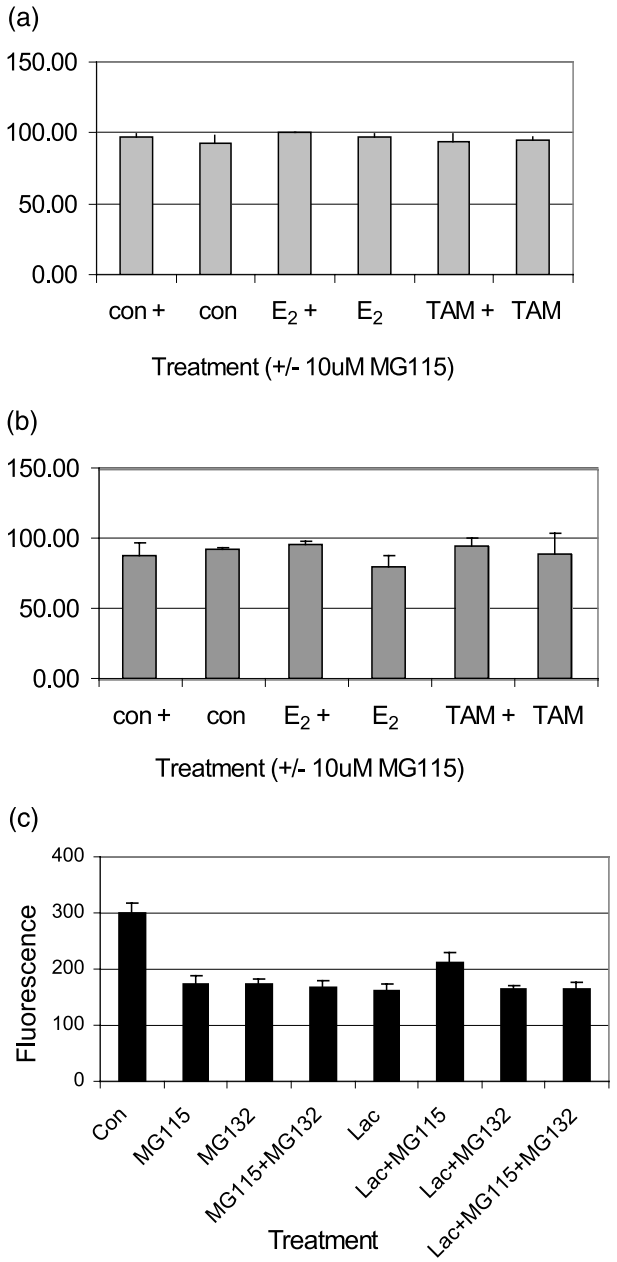

Figure 3 Effect of proteasome inhibitor (MG115; 10^{-6} M) 1 h pretreatment on cell viability 3 h following dosing with 10^{-8} M E_2 or 10^{-6} M TAM. (a) MCF-7 cell viability, (b) Ishikawa cell viability and (c) representative graph of proteasome activity in MCF-7 cells dosed with various proteasome pathway inhibitors (10^{-6} M MG 115, MG132 or lactacystin; Sigma) and lysed after 4 h. Fluorescence levels produced by the hydrolysis of Suc-LLVY-AMC were assayed. Values are expressed as percentage viability (a and b) formulated from mean values±S.E. of three experiments. Con, control; lac, lactacystin.

these cells with the MG115 proteasome inhibitor resulted in a large increase in ERβ protein levels (Fig. 4d). The effect of TAM as ligand for ERβ was assessed in both cell lines. In MCF-7 cells, treatment with TAM led to an increase of ERβ protein suggesting that, as with ERα, this compound may stabilise the receptor (Fig. 4c). Western blot analysis of TAM-treated Ishikawa cells showed a loss of ERβ protein; pretreatment with the proteasome inhibitor resulted in an increase of ERβ protein above control level (Fig. 4d).

Confocal microscopy

Immunocytochemical detection of ERα and ERβ by confocal microscopy in the MCF-7 cells demonstrated that ERα was detectable primarily in the nucleus, as indicated by Hoechst staining; there was also cytoplasmic localisation of the protein. ERβ was also located predominantly in the nucleus (Fig. 5a, b and inset). However, in the Ishikawa cells, ERα was detected principally in the cytoplasm while ERβ was located mainly in the nucleus (Fig. 5c and d). Treatment of MCF-7 cells with MG115 alone (Fig. 5f) resulted in a greater nuclear ERα immunoreactivity relative to vehicle-treated controls (Fig. 5e). Treatment with E_2 (Fig. 5 g) resulted in loss of ERα signal relative to controls and this was prevented by pretreatment with MG115 (Fig. 5 h). TAM treatment increased the levels of nuclear staining for ERα (Fig. 5i) but this immunoreactivity was not further enhanced by pretreatment with MG115 (Fig. 5j). In MCF-7 cells, ERβ was much less responsive to pretreatment with MG115 (Fig. 5 l) relative to controls (Fig. 5k). In contrast, we were unable to detect changes in staining intensities of ERα (or ERβ) protein in the Ishikawa cells by the immunostaining methods employed following MG115, E_2 or TAM treatments (data not shown).

RT-PCR analysis of ERα and ERβ mRNA levels following E_2 and TAM treatment

We wanted to exclude the possibility that the effects we observed on ER protein levels were due to regulation of mRNA. ERα mRNA levels were not significantly altered in either cell line after a 3-h incubation with either E_2 or TAM relative to the GAPDH housekeeping gene (Fig. 6a and b). However, a significant increase in ERβ mRNA compared with control ($P \leq 0{\cdot}05$) was observed following 3 h of treatment with E_2 in the MCF-7 cell line, conversely TAM led to a decrease in ERβ mRNA (Fig. 6c and d). Pretreatment of the cells with MG115 did not influence changes in the mRNA levels with either compound and consequently the mechanisms responsible were not investigated further.

E_2 activates non-genomic signalling via Akt in MCF-7 cells

We investigated the potential of E_2 and TAM to activate ER-initiated non-genomic signalling pathways, and any

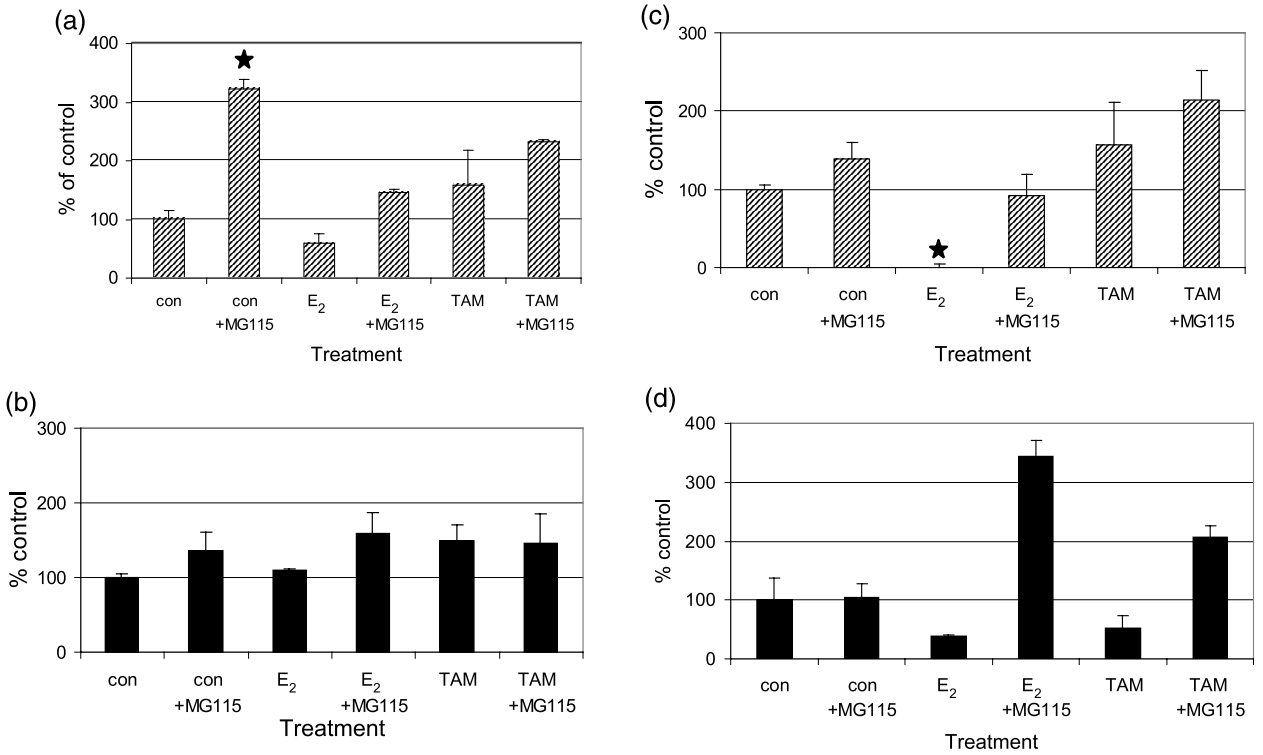

Figure 4 Western blot analysis of (**a**) ERα protein expression in MCF-7 and (**b**) Ishikawa cells. ERβ expression in MCF-7 (**c**) and Ishikawa (**d**) cells. Cells were pretreated for 1 h with 10^{-6} M MG115 and then dosed for 3 h with 10^{-8} M E_2 or 10^{-6} M TAM. Whole cell lysates were prepared after a total of 4 h. ER expression is expressed as percentage of control formulated from mean values normalised to GAPDH expression ±S.E. *$P \leq 0{\cdot}05$ (ANOVA). Con, control.

subsequent links to altered ratios of ERα:ERβ proteins levels by inactivating the proteasome pathway. MCF-7 lysates showed a significant increase in Akt phosphorylation by 30 min following dosing of the cells with E_2 ($P \leq 0{\cdot}05$; Fig. 7a). This significant increase in Akt phosphorylation was blocked by pretreatment of the cells with 50μM LY2940002 PI3K inhibitor, confirming that Akt was phosphorylated by PI3K in response to E_2. Pretreatment of the cells with the proteasome pathway inhibitor MG115 did not affect activation of the Akt pathway. In the Ishikawa cells, Akt phosphorylation was not significantly increased (Fig. 7b). Western blot analysis of ERα protein levels within nuclear and cytosolic/cell membrane fractions from MCF-7 cells showed that they have detectable levels of the protein in both fractions.

Several conflicting reports have been published as to whether proliferation of MCF-7 cells stimulated by E_2 is coupled to phosphorylation of Erk1/2 and activation of the MAP kinase (MAPK) non-genomic signalling pathway. We assessed Erk1/2 phosphorylation levels in both cell types. No increase in phosphorylation of Erk1/2 was observed in MCF-7 cells following E_2 or TAM treatment (Fig. 8a). However, rapid activation of the pathway was observed when MCF-7 cells were treated with epidermal growth factor (EGF), a well-documented activator of MAPK (data not shown). Interestingly, phosphorylation of Erk1/2 is significantly induced in the Ishikawa cell type, 30 min after treatment with E_2, but only in cells with an MG115-inhibited proteasome pathway ($P \leq 0{\cdot}05$; Fig. 8b). When cells were incubated with 30μM PD98059, MEK inhibitor phosphorylation of Erk1/2 was abolished confirming that MEK was responsible for the phosphorylation of Erk1/2 in response to E_2 treatment.

Discussion

The aim of this study was to investigate the role of ligand-mediated degradation and transcription activation pathways in ER subtype-mediated action of tamoxifen and oestradiol, comparatively between breast and uterine cancer cells. We have shown that the ERβ protein is targeted for degradation following oestradiol binding in the MCF-7 breast carcinoma cell type and that inhibition of the proteasome pathway stops this loss of protein (Fig. 4c). Our results have suggested that this is independent of ER gene transcription as the loss of protein was not accompanied by a decrease in mRNA

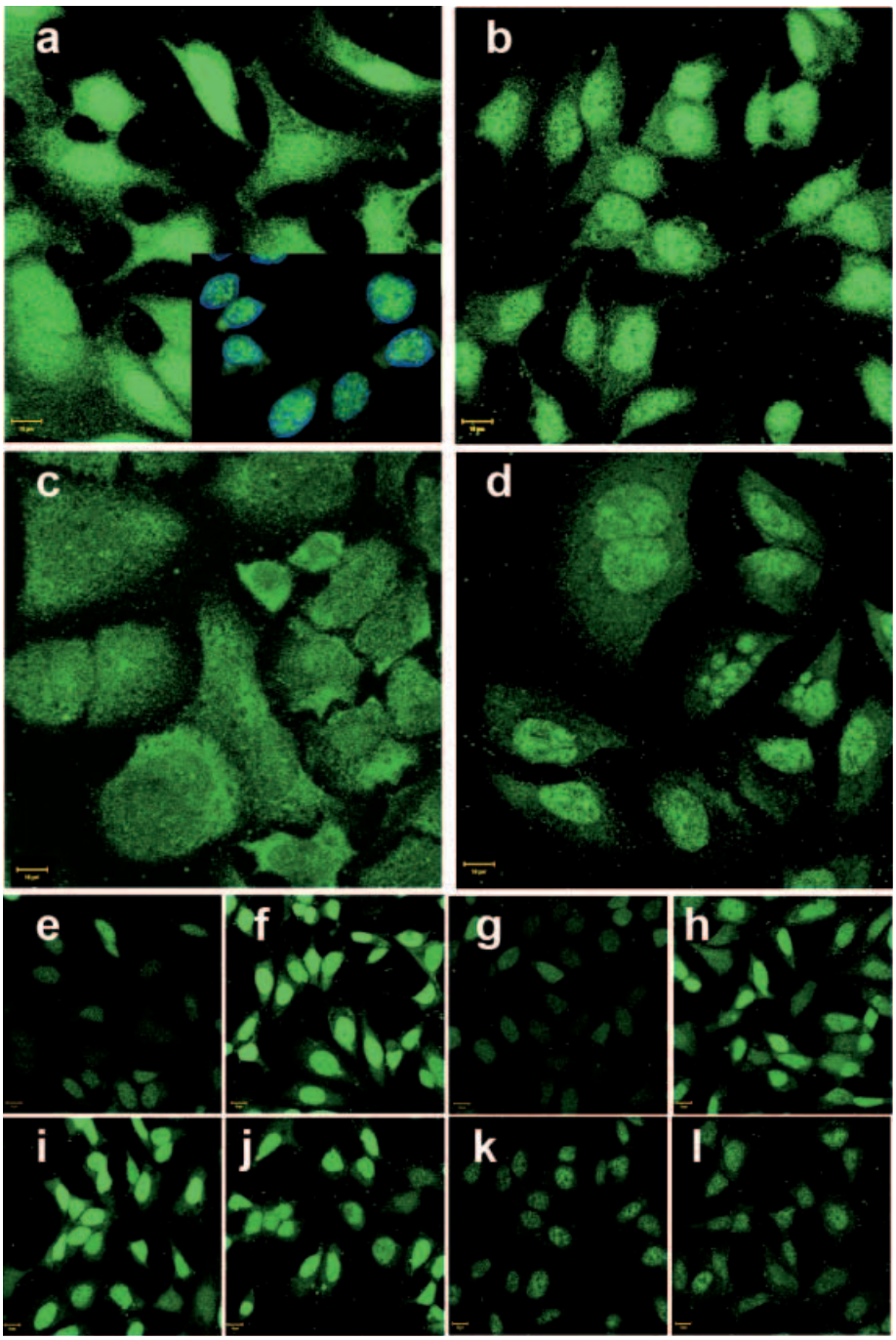

Figure 5 Confocal immunohistochemical localisation of ERα and ERβ in MCF-7 and Ishikawa cells showing the effects of 1 h pretreatment with 10^{-6} M MG115 followed by 3 h 10^{-8} M E_2 or 10^{-6} M TAM. Cells were grown for 72 h in chamber slides at 37 °C in 5% CO_2 in air in charcoal-stripped medium before dosing and fixing in methanol at −20 °C as described in Materials and methods. ERα and ERβ were detected with mouse monoclonal primary antibodies and anti-mouse Alexa 488-conjugated secondary antibody. Hoechst 33342 dye was subsequently added for nuclear staining. Pictures were taken using standardised photomultiplier gain settings. The bars represent 10 μm. (a and b) MCF-7 cells and (c and d) Ishikawa cells. (a and c) ERα and (b and d) ERβ. Inset in (a) shows ERα (green) predominantly co-localising with Hoechst nuclear staining (blue). (e–j) ERα in MCF-7 cells showing the effect of pretreatments: (e) vehicle control, (f) MG115 alone, (g) E_2, (h) MG115 pretreatment followed by E_2, (i) TAM and (j) MG115 pretreatment followed by TAM. (k and l) ERβ in MCF-7 cells: (k) vehicle control and (l) pretreated with MG115 alone. These images are representative of three separate experiments.

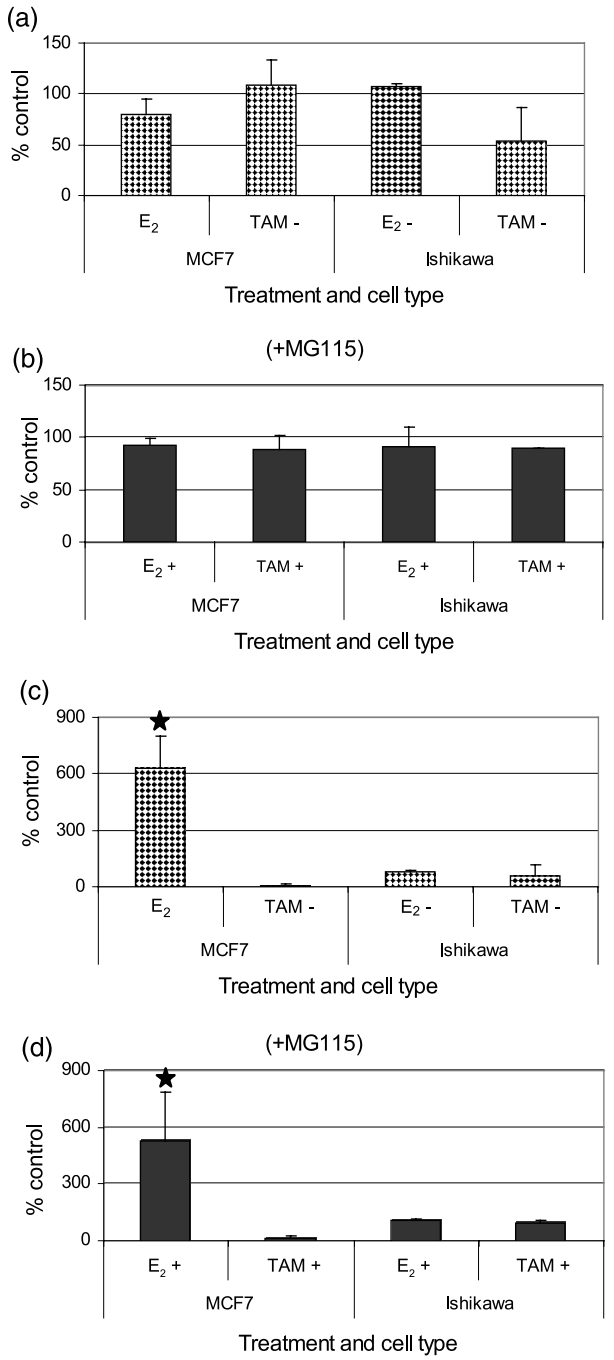

Figure 6 RT-PCR analysis of (a and b) ERα and (c and d) ERβ mRNA expression. Cells were pretreated for 1 h with 10^{-6} M MG115 and dosed for 3 h with 10^{-8} M E_2 or 10^{-6} M TAM and mRNA was extracted by oligo dT-linked Dynabeads after a total of 4 h. ER mRNA expression is expressed as percentage of control formulated from mean values±S.E. **P*≤0·05 (ANOVA).

and, in fact, a significant increase of ERβ mRNA was observed in line with previous findings (Cappelletti *et al.* 2003) (Fig. 6c). Oestradiol-induced proteasome-mediated ERβ degradation has been described in human vascular endothelial cells (Tschugguel *et al.* 2003) and in human aortic smooth muscle cells (Barchiesi *et al.* 2004). A further study where MCF-7 cells were transiently transfected with an ERβ expression plasmid showed that the proteasome inhibitor lactacystin had no effect on the marginal oestradiol-induced ERβ degradation observed (Peekhaus *et al.* 2004). However, although the degradation pathways involved in the control of endogenous ERβ cannot be directly compared with those created by over-expression of the ERβ expression protein in this cell model, the data suggested that the proteasome pathway must be inhibited before treatment of the cells with oestradiol, a state not investigated in the Peekhaus *et al.* (2004) study. The results of our study have suggested that ERβ is also a target for the proteasome in human breast cancer-derived cells. By both western blot and immunocytochemical staining methods, we have confirmed that ERα is targeted for degradation via the proteasome pathway on binding oestradiol, but not tamoxifen, in the MCF-7 cell line (Figs 4a and 5 g and i) (Wijayaratne & McDonnell 2001).

A key finding of this study was that unliganded ERα protein was found to increase in MCF-7 cells with an inhibited proteasome pathway; this was not found for ERβ (Figs 4a and c and 5f). These findings confirmed that ERα is a target for the proteasome pathway in an unliganded state and, in the absence of an active proteasome and ligand, ERα is ubiquitinated and immobilised in the nuclear matrix (Reid *et al.* 2003). It is interesting to note that in this instance no increase in ERβ protein was observed; we suggest that, unlike ERα, ERβ is not a target for the proteasome in an unliganded state in the MCF-7 cell type. Tamoxifen did not lead to reduced levels of ERα or ERβ proteins in the MCF-7 cell type, suggesting that tamoxifen binding stabilises both the receptors, in line with the findings of others (Wijayaratne & McDonnell 2001, Preisler-Mashek *et al.* 2002, Tschugguel *et al.* 2003) (Fig. 4a and c). It is of note that tamoxifen, although leading to an increase in ERβ protein, led to a decrease in ERβ mRNA, providing evidence for the hypothesis that this compound stabilises the protein and does not increase protein synthesis. Tamoxifen and E_2 have opposite effects on ER subtype stability in the breast cell line investigated, supporting tamoxifen's role as an anti-oestrogen in breast cancer.

In this study, E_2 and tamoxifen have analogous effects on ER degradation in the uterine cell type. We have demonstrated for the first time that tamoxifen induces ligand-mediated degradation of ERβ protein, an effect requiring an active proteasome (Fig. 4d). In other cell lines, tamoxifen increases ER stability (Lonard & Smith 2002, Marsaud *et al.* 2003, Peekhaus *et al.* 2004). Increasing the ERβ:ERα ratio by transfection in T47D cells reduces ERα-mediated proliferation (Strom *et al.*

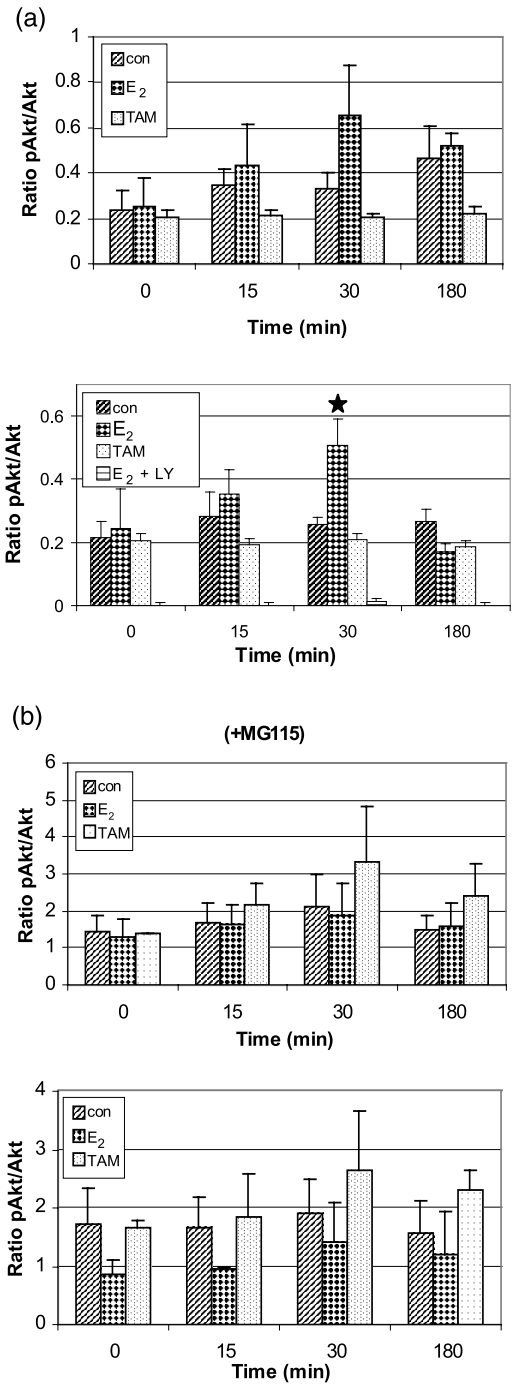

Figure 7 Representative graphs and western blot analysis of Akt and phosphorylated Akt protein levels in (a) MCF-7 and (b) Ishikawa cells. Cell lines were maintained in phenol red-free DMEM/F12 or RPMI containing 10% DCST-FCS for 72 h, pretreated with 10^{-6} M MG115 or 50μM LY2940002 for 1 h before dosing with 10^{-8} M E_2 or 10^{-6} M TAM. Whole cell lysates were prepared at 0, 15, 30 and 180 min. Protein expression is expressed as a ratio of pAkt/Akt normalised to GAPDH expression from mean values±S.E. of three experiments. *$P \leq 0{\cdot}05$ (ANOVA).

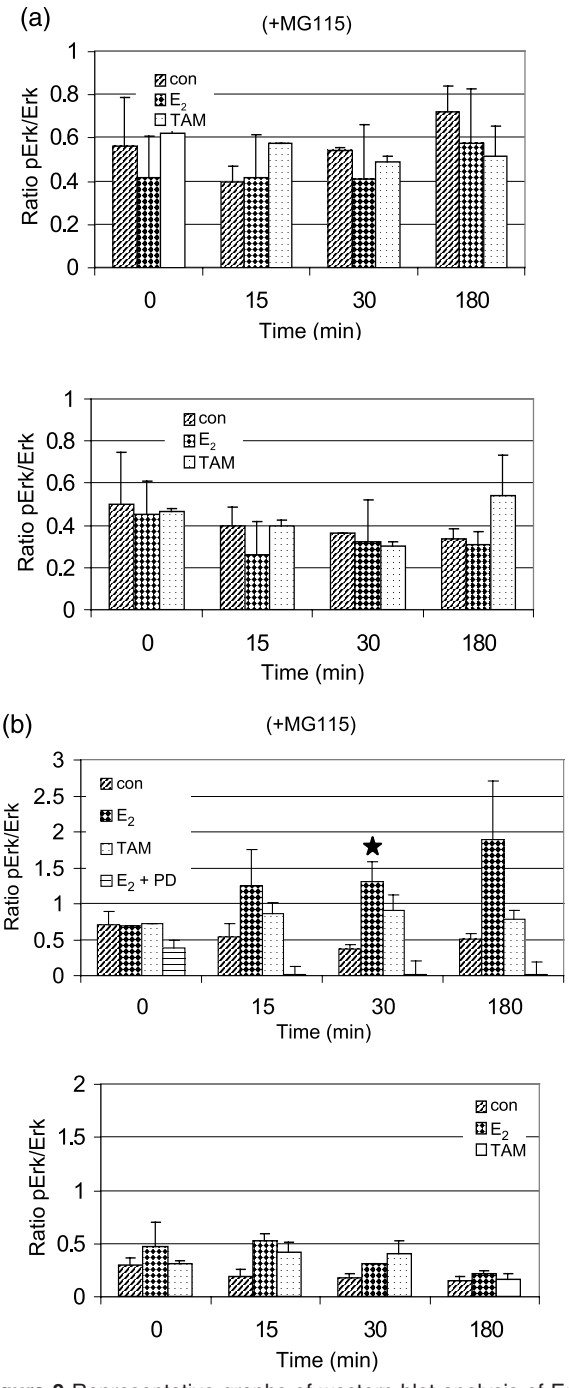

Figure 8 Representative graphs of western blot analysis of Erk and phosphorylated Erk (pERk) protein levels in (a) MCF-7 and (b) Ishikawa cells. Cell lines were maintained in phenol red-free DMEM/F12 or RPMI containing 10% DCST-FCS for 72 h, pretreated with 10^{-6} M MG115 for 1 h before dosing with 10^{-8} M E_2 or 10^{-6} M TAM. 30μM PD98059 (PD) was used to inhibit E_2-induced Erk phosphorylation in the Ishikawa cell type. Whole cell lysates were prepared at 0, 15, 30 and 180 min. Protein expression is expressed as a ratio of pErk/Erk normalised to GAPDH expression from mean values±S.E. of three experiments. *$P \leq 0{\cdot}05$ (ANOVA).

2004); we suggest that tamoxifen's ability to down-regulate ERβ may play a role in the proliferative effects of the drug in the human uterus (Tomas *et al.* 1995, Goldstein 2001). E_2 induces ligand-mediated degradation of ERβ thus reducing the ERβ:ERα ratio in this cell type, allowing ERα-mediated proliferation to proceed. Interestingly, Wijayaratne & McDonnell (2001) demonstrated that tamoxifen could induce degradation of a mutant ERβ protein unable to bind DNA, suggesting that the DNA-binding capacity of the receptor was necessary for stabilisation of the protein by this ligand. As observed in the MCF-7 cells, unliganded ERβ proteins are not subject to proteasomal degradation, suggesting that a ligand-induced conformational change is required for ubiquitination to occur (Fig. 4d).

These converse effects of oestradiol and tamoxifen on ER subtype degradation are in line with the hypotheses that the classical model for ER signalling is oversimplified, and various other cell-specific co-activators and co-repressors bind to and influence activation and degradation of the ligand-bound receptors within different target cell types (Nawaz *et al.* 1999, Lonard *et al.* 2000, McKenna & O'Malley 2001, Uchikawa *et al.* 2003). It is clear that ligand-induced conformational changes, allowing interaction with cell-specific cofactors, are key in target cell response to oestrogen and SERMs. Further characterisation studies are required to determine which proteins are central components in this pathway and their expression levels within the two cell types.

To further evaluate the effect of E_2 and tamoxifen on the transcriptional activities of the ERs we investigated the involvement of ER in the activation of pathways leading to phosphorylation of the Akt and Erk proteins in both breast and uterine cell lines. Recent studies have described rapid non-genomic actions of ER stimulated by E_2 leading to a substantial increase in pAkt and cell proliferation in MCF-7 (Marquez & Pietras 2001) and vascular endothelial cells (Pedram *et al.* 2002). Integration of non-genomic and genomic activation of ERα has been described, involving oestrogen activation of the PI3K/Akt pathway through ERα, terminating in interaction with nuclear ERα altering its expression and activity in MCF-7 cells (Stoica *et al.* 2003). Following confirmation that in MCF-7 cells E_2 treatment rapidly increases pAkt levels, we have shown that tamoxifen does not stimulate phosphorylation of Akt (Fig. 7b). We propose that tamoxifen's inability to activate the PI3K/Akt signalling pathway in MCF-7 cells may contribute to its inhibition of cell proliferation. E_2 or tamoxifen did not activate phosphorylation of Akt in Ishikawa cells, suggesting that classical genomic activation of transcription is responsible for the proliferative effects of these compounds in the uterine cell type.

In contrast to Akt, no increase in phosphorylation of Erk1/2 was observed following E_2 or tamoxifen treatment of MCF-7 cells (Fig. 8a) confirming previous reports (Lobenhofer & Marks 2000, Caristi *et al.* 2001) but conflicting with others (Song *et al.* 2002). We confirmed that the MAPK/Erk signalling cascade could be activated in our MCF-7 cell model by EGF, a well-documented activator of the MAPK cascade, and therefore suggest that varying experimental conditions including culture media, cell type variation and sensitivity of detection methods could explain this discrepancy. We have demonstrated for the first time E_2-induced phosphorylation of Erk1/2 in the Ishikawa cell type only in the absence of an active proteasome pathway (Fig. 8b). We have shown that ERβ is rapidly degraded via the proteasome pathway in response to E_2, and an excess of ERβ protein levels within the cell, in the presence of MG115, may lead to increased activation of Erk1/2 phosphorylation in this cell type. Further investigations are required to determine the role of ERβ in non-genomic signalling, although evidence from the ERKO mouse model describes oestrogen-induced activation of the MAPK signalling cascade, suggesting a role for ERβ (Singh *et al.* 2000).

In conclusion, our data have demonstrated tamoxifen and E_2 control of ER subtype stability and that non-genomic activation of transcription pathways is cell specific.

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