Tumour necrosis factor and PI3-kinase control oestrogen receptor alpha protein level and its transrepression function

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Oestrogen receptor alpha (ERα) is an oestrogen-activated transcription factor, which regulates proliferation and differentiation of mammary epithelial cells by activating or repressing gene expression. ERα is a critical prognostic indicator and a therapeutic target for breast cancer. Patients with tumours that express higher level of ERα have better prognosis than patients with tumours that are ERα negative or express lower level of ERα. Better prognosis in ERα-positive patients is believed to be due to repression of proinvasive gene expression by ERα. Oestrogen receptor alpha represses gene expression by transrepressing the activity of the transcription factors such as nuclear factor-kappaB or by inducing the expression of transcriptional suppressors such as MTA3. In this report, we show that ERα transrepresses the expression of the proinvasive gene interleukin 6 (IL-6) in ERα-negative MDA-MB-231 breast cancer cells stably overexpressing ERα. Using these cells as well as ERα-positive MCF-7 and ZR-75-1 cells, we show that tumour necrosis factor alpha (TNFα) and the phosphatidylinositol-3-kinase (PI3-kinase) modulate transrepression function of ERα by reducing its stability. From these results, we propose that TNFα expression or PI3-kinase activation lead to reduced levels of ERα protein in cancer cells and corresponding loss of transrepression function and acquisition of an invasive phenotype.

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Oestrogen receptor alpha (ERα) expression status is of prognostic significance for breast cancer. Breast cancer patients with the highest levels of ERα protein have a 90% 5-year survival rate and display very few p53 mutations. Patients with lower ERα levels have ~45% 5-year survival and higher p53 mutation rates. The survival rate in these patients is similar to patients with a subset of ERα-negative breast cancer (Sorlie et al, 2001). Better prognosis in ERα-positive breast cancer patients can partly be attributed to their response to antihormone therapy (Ali and Coombes, 2002). However, because patients with higher rather than lower ERα protein have better prognosis, it is likely that some of the ERα-regulated genes suppress invasion and metastasis of breast cancer. Consistent with this possibility, it was shown recently that ERα-dependent expression of metastasis associated protein 3 (MTA3) is required to prevent invasive growth of breast cancer cells (Fujita et al, 2003). Furthermore, a recent microarray study has shown that among ~400 genes regulated by ERα/oestrogen in MCF-7 cells, majority of them (70%) are downregulated (Frasor et al, 2003). Some of the downregulated genes are known to be involved in invasion and homing of metastatic cancer cells (Muller et al, 2001).

Oestrogen receptor alpha is an oestrogen-activated transcription factor, which modulates gene expression by binding to oestrogen response elements (ERE) in the responsive promoter and through protein–protein interactions (Mangelsdorf et al, 1995; Di Croce et al, 1999). Oestrogen receptor alpha contains a central DNA binding domain (DBD), C-terminal ligand binding domain (LBD), as well as ligand-dependent activation function (AF-2) and N-terminal ligand-independent activation function (AF-1). Upon binding to oestrogen, ERα homodimers bind to ERE in the responsive gene promoters and activate gene expression. In addition, ERα homodimers activate non-ERE containing promoters by interacting with transcription factors such as SP-1 and AP-1 (Gaub et al, 1990; Paech et al, 1997; Dong et al, 1999). Transactivation by ERα involves ligand-dependent recruitment of coactivators, which serve as an intermediate between the receptor and the RNA polymerase II transcription complex (Horwitz et al, 1996; Glass and Rosenfeld, 2000). Although binding of oestrogen to LBD is essential for complete activation of ERα, phosphorylation by extracellular signal-activated kinases is thought to play a role in oestrogen-dependent and oestrogen-independent activity of ERα (Ali et al, 1993; Le Goff et al, 1994). Recently, a novel cell-type
specific nongenomic action of ER\textsubscript{\textalpha} involving oestrogen-dependent association of ER\textsubscript{\textalpha} with phosphatidylinositol-3-kinase (PI3-kinase) leading to activation of the cell survival kinase AKT has also been reported (Simoncini et al, 2000). In addition, ER\textsubscript{\textalpha} localised in the plasma membrane has been shown to activate the MAP kinase pathway and contribute to growth regulation of breast cancer cells (Filardo et al, 2000; Marquez and Pietras, 2001; Razandi et al, 2003).

Transrepression of gene expression through protein–protein interaction is also a critical function of ER\textsubscript{\textalpha}. For example, inhibition of GATA-1-mediated transcription by ER\textsubscript{\textalpha} is responsible for suppression of erythroid differentiation by oestrogen (Blobel et al, 1995). The protective effect of oestrogen against sepsis is believed to be due to the suppression of proinflammatory gene expression (Schroder et al, 1998; Evans et al, 2002). Similarly, ER\textsubscript{\textalpha}-dependent repression of nuclear factor kappa B (NF-κB) activity is important for maintaining bone density (Jilka et al, 1992; Pottratz et al, 1994; Stein and Yang, 1995). Evans et al (2002) have identified several NF-κB-regulated genes that are repressed by ER\textsubscript{\textalpha}, which include antiapoptotic proteins GADD45\textbeta, apoptosis inhibitor 2, A20 and NF-κB p105. A recent report suggested that ER\textsubscript{\textalpha} at higher levels reduces cancer cell growth and angiogenesis by inhibiting the expression of vascular endothelial growth factor (Ali et al, 2001). Furthermore, it was reported that unliganded and liganded ER\textsubscript{\textalpha} reduce cancer cell migration and invasion, through a mechanism that involves protein–protein interaction (Platet et al, 2000). Unlike the case of the glucocorticoid receptor (GR) where transrepression function is well characterised (Nissen and Yamamoto, 2000), the mechanism of ER\textsubscript{\textalpha}-mediated transrepression is not completely understood. Antagonism of NF-κB activity has been used as a model system to understand ER\textsubscript{\textalpha}-mediated transrepression. Repression of NF-κB activity by ER\textsubscript{\textalpha} is cell type specific (Cerillo et al, 1998). Both the DBD and LBD of ER\textsubscript{\textalpha} are essential for efficient repression of NF-κB activity (Stein and Yang, 1995; Valentine et al, 2000). It is suggested that ER\textsubscript{\textalpha} interacts directly with the Rel-homology domains (RHD) of the NF-κB subunits, p50 and p65, thereby interfering with the transcriptional activity of DNA-bound NF-κB (Stein and Yang, 1995). Apart from inhibition through direct protein–protein interaction, competition for the limiting amount of common coactivators such as SRC-1 and p300/CBP is suggested to play a role in ER\textsubscript{\textalpha}-dependent repression of NF-κB activity, although studies with ER\textsubscript{\textalpha} harbouring mutations in its transactivation domain fail to support such a mechanism (Sheppard et al, 1999; Harnish et al, 2000; Valentine et al, 2000).

The goals of this study were to determine whether ER\textsubscript{\textalpha} transrepresses the expression of interleukin 6 (IL-6), a cytokine that is linked to breast cancer cell invasion and motility as well as resistance to chemotherapy (Tamm et al, 1991; Conze et al, 2001), and to identify signalling pathways that may modulate transrepression by altering the stability of ER\textsubscript{\textalpha}. Using the ER\textsubscript{\textalpha}-negative breast cancer cell line MDA-MB-231 stably overexpressing ER\textsubscript{\textalpha}, we show that ER\textsubscript{\textalpha} transrepresses tumour necrosis factor alpha (TNF\textalpha)-inducible expression of IL-6. We also show that TNF\textalpha and PI3-kinase pathway modulate transrepression by reducing the stability of ER\textsubscript{\textalpha}.

MATERIALS AND METHODS

Generation of ER\textsubscript{\textalpha}-overexpressing cells

MCF-7, ZR-75-1 and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cDNA encoding ER\textsubscript{\textalpha} was cloned into the EcoRI site of the retroviral vector LxSN (pLxSN-ER\textsubscript{\textalpha}) (Miller and Rosman, 1989). Packaging of retrovirus and infection of MDA-MB-231 were performed as described previously (Newton et al, 1999). Briefly, AM12 cells were transfected with pLxSN or pLxSN-ER\textsubscript{\textalpha} expression vector and selected in media containing 600 μg ml\textsuperscript{-1} G418. G418-resistant colonies were pooled and media supernatant with virus was used for infecting MDA-MB-231 cells. MDA-MB-231 cells were incubated with viral supernatant for 2 h in the presence of 8 μg ml\textsuperscript{-1} polybrene. The transduced cells were grown in the presence of G418 (1 mg ml\textsuperscript{-1}). Individual G418-resistant colonies were isolated and ER\textsubscript{\textalpha} expression was measured by Western blotting. The constitutively active PI3-kinase expression vector (myr-PI 3-Kp110) was purchased from Upstate Biotechnology (Charlottesville, VA, USA). Constitutively active AKT (CA-AKT) and kinase-dead AKT (KD-AKT) have been described previously (Campbell et al, 2001). Cells were transfected with expression vectors using Lipofectamine 2000 transfection reagent as recommended by the manufacturer and analysed for ER\textsubscript{\textalpha} protein levels 48 h after transfection (Invitrogen, Carlsbad, CA, USA).

Northern blot analysis

Total RNA was prepared using the RNAeasy kit from Qiagen (Valencia, CA, USA). RNA was subjected to Northern blot analysis as previously described (Newton et al, 1999). Interleukin-6 CDNA was purchased from ATCC, whereas tumour necrosis factor receptor associated protein 1 (TRA1-1) CDNA has been described previously (Rothe et al, 1994).

Western blot analysis

Whole-cell extracts were prepared in radioimmunoassay buffer (RIPA; 50 mM Tris pH 7.5, 0.25% sodium deoxycholate, 1% NP40, 150 mM NaCl, 1 mM EDTA, 100 μM sodium orthovanadate, 1 mM sodium fluoride, 1 mM β-glycerophosphate, 0.5 mM PMSF, 2 μg ml\textsuperscript{-1} each of aprotenin, leupeptin and pepstatin) and subjected to Western blot analysis as previously described (Newton et al, 1999). Oestrogen receptor alpha antibody raised against B-domain of ER\textsubscript{\textalpha} was purchased from Chemicon (MAB463; Temecula, CA, USA), whereas β-actin antibody was from Sigma Chemicals (St Louis, MO, USA). MG132, PD98059, LY294002, PP2 and protein kinase A (PKA) inhibitor peptide were purchased from Calbiochem (San Diego, CA, USA), whereas TNF\textalpha was purchased from R&D systems (Minneapolis, MN, USA). Cells were incubated with kinase inhibitors for 2 h before addition of TNF\textalpha. 4-Hydroxytamoxifen was purchased from Sigma Chemicals, whereas ICI182780 was purchased from Tocris (Ellisville, MO, USA).

RESULTS

ER\textsubscript{\textalpha} reduces TNF\textalpha-inducible IL-6 but not TRAF-1 expression

Previously, we reported constitutive NF-κB activation in the ER\textsubscript{\textalpha}-negative breast cancer cell line MDA-MB-231, which correlated with increased expression of several NF-κB-inducible genes including IL-6, Mn-SOD, cIAP-2 and TRAF-1 (Patel et al, 2000). In addition, using transient transfection assays, we showed transrepression of NF-κB activity by ER\textsubscript{\textalpha} in these cells (Nakashtri et al, 1997). To further characterise the transrepression function of ER\textsubscript{\textalpha}, we stably overexpressed ER\textsubscript{\textalpha} in MDA-MB-231 cells using retrovirus-mediated gene transfer (Figure 1A). Oestrogen receptor alpha-3 and ER\textsubscript{\textalpha}-6 clones overexpress wild-type ER\textsubscript{\textalpha}, whereas ER\textsubscript{\textalpha}-8 and ER\textsubscript{\textalpha}-9 overexpress mutant ER\textsubscript{\textalpha} (C530R), which cannot activate transcription of an ERE-containing reporter gene (data not shown). Cysteine 530 is within the recently identified KCK motif involved in intramolecular AF-1 and AF-2 interaction and this mutation reduces the affinity of ER\textsubscript{\textalpha} to oestrogen (E2) (Metivier et al, 2002). Cells expressing the mutant protein were used to evaluate transrepression independent of coactivator competition and by a mutant ER\textsubscript{\textalpha} with reduced affinity to E2. We compared the
This could be due to sequestration and subsequent degradation of various ligands on basal and TNF-α expression was not influenced by E2. TNF-α and TRAF-1 expression is lower in ERα compared to LxSN2 cells (Figure 2B). Oestrogen receptor alpha-6 cells were treated with E2 (10 nM), tamoxifen (1 μM) or ICI182780 (100 nM) for 2 h followed by further incubation for 16 h with or without TNFα. Oestrogen receptor alpha-protein level was determined by Western blotting. The same blot was reprobed for β-actin. (B) ICI182780 overcomes the transrepression function of ERα. RNA from cells treated as above was subjected to Northern analysis with IL-6 or TRAF-1 probe. Two exposures of the IL-6 blot are shown to highlight the effect of E2 on the basal IL-6 expression level in ERα-6 cells.

Pure antioestrogen ICI182780 reverses transrepression function of ERα

To further confirm the role of ERα in reducing TNFα-inducible expression of IL-6 in ERα-overexpressing cells, we preincubated cells with E2, tamoxifen or ICI182780 for 2 h and measured IL-6 expression with or without TNFα treatment for 16 h. Binding of E2 leads to activation and subsequent proteosome-dependent degradation of ERα (Lonard et al, 2000). Previous studies have shown that binding of tamoxifen to ERα leads to stabilisation, whereas binding to ICI182780 leads to degradation of ERα without activation (Ali et al, 1993). Tamoxifen stabilised, whereas E2 and ICI182780 reduced ERα level in ERα-6 cells (Figure 2A). The effect of various ligands on basal and TNFα-inducible IL-6 and TRAF-1 expression was examined. Oestrogen reduced basal IL-6 expression in ERα-6 cells compared to LxSN2 cells (Figure 2B, middle panel). This could be due to sequestration and subsequent degradation of common coactivators by ERα. Interestingly, TNFα-inducible IL-6 expression was not influenced by E2. TNFα-induced IL-6 expres-

TNFα reduces the stability of ERα protein

We consistently observed a lower level of ERα in TNFα-treated cells compared to untreated cells and a further enhancement of ICI182780-dependent degradation of ERα by TNFα (Figure 2A). In early passage cells, TNFα reduced ERα protein level by as much as 60% (data not shown). This raised the possibility that TNFα modulates transrepression function of ERα by inducing its degradation. Towards this end, ERα-6 and ERα-8 cells with or without prior treatment with TNFα for 16 h were incubated with cyclohexamide to block protein synthesis. Cells were harvested at specific time intervals and ERα protein was measured by Western blotting. Oestrogen receptor alpha stability was much lower in cells pretreated with TNFα compared to untreated cells (Figure 3A). Oestrogen receptor alpha undergoes proteosome-mediated degradation in TNFα-treated cells as the proteosomal inhibitor MG132 prevented ERα degradation (Figure 3B). Neither caspase inhibitors nor calpain inhibitors altered the stability of ERα under untreated and TNFα-treated conditions (data not shown). Oestrogen receptor

Figure 1 Oestrogen receptor alpha reduces TNFα-inducible IL-6 but not TRAF-1 expression in MDA-MB-231 cells. (A) Oestrogen receptor alpha expression in MDA-MB-231 cells. Oestrogen receptor alpha expression in cells transduced with retrovirus without the ERα coding sequence (LxSN2 and LxSN23) or with the ERα coding sequence (ERα-3, ERα-6, ERα-8 and ERα-9) was measured by Western blotting. Note that ERα-3 and ERα-6 cells express the wild-type receptor, whereas ERα-8 and ERα-9 cells express mutant receptor. (B) TNFα-inducible IL-6 but not TRAF-1 expression is lower in ERα-overexpressing cells compared to control cells. Cells were treated with TNFα for the indicated times and IL-6 or TRAF-1 expression was measured by Northern blot analysis. The same blot was reprobed with ribosomal protein gene 36B4 to ensure equal loading.

Figure 2 Pure antioestrogen ICI182780 overcomes the transrepression function of ERα. (A) Effect of oestrogen (E2), tamoxifen and ICI182780 on the stability of ERα. Oestrogen receptor alpha-6 cells were treated with E2 (10 nM), tamoxifen (1 μM) or ICI182780 (100 nM) for 2 h followed by further incubation for 16 h with or without TNFα. Oestrogen receptor alpha protein level was determined by Western blotting. The same blot was reprobed for β-actin. (B) ICI182780 overcomes the transrepression function of ERα. RNA from cells treated as above was subjected to Northern analysis with IL-6 or TRAF-1 probe. Two exposures of the IL-6 blot are shown to highlight the effect of E2 on the basal IL-6 expression level in ERα-6 cells.
alpha transcript levels were similar in untreated and TNFα-treated cells, suggesting that the effect of TNFα on ERα is at the level of protein stability (Figure 3C). None of the effects of TNFα on ERα is due to TNFα-induced apoptosis of MDA-MB-231 as these cells were resistant to TNFα irrespective of ERα overexpression (data not shown). Tumour necrosis factor alpha-induced destabilisation of ERα was not restricted to MDA-MB-231 cells as TNFα induces the degradation of ERα-positive MCF-7 and ZR-75-1 cells (Figure 3D). Consequences of ERα degradation on transcription in MCF-7 cells could not be studied because of lack of IL-6 expression in these cells and their sensitivity to TNFα-induced apoptosis (data not shown).

Phosphatidylinositol-3-kinase inhibitor LY294002 stabilises ERα protein in MDA-MB-231 cells and inhibits TNFα-induced but not E2-induced degradation of ERα in MCF-7 cells

MAPK, cyclin A/cdk2, AKT, RSK2, PKA, PKA, p38 kinase and Src phosphorylate ERα (Ali et al., 1993; Lee and Bai, 2002; Wang et al., 2002). Phosphorylation leads to ligand-independent activation in most cases, and activated ERα undergoes coactivator-ubiquitin-dependent degradation (Lonard et al., 2000). Phosphatidylinositol-3-kinase, which is upstream of AKT, as well as MAPK are constitutively active in MDA-MB-231 cells and may promote phosphorylation-dependent degradation of ERα (Ma et al., 2001; Sliva et al., 2002). Consistent with this possibility, ERα showed ligand-independent activity in ERα-3 and ERα-6 cells (data not shown). To investigate whether any of these kinases determine the stability of ERα and thus modulate transcription function, we treated cells with various inhibitors for 16 h with or without TNFα treatment and measured ERα protein. The MAP kinase inhibitor PD98059, PKA inhibitory peptide or Src kinase inhibitor did not alter ERα protein level in ERα-6 cells (Figure 4A). In contrast, the PI3-kinase inhibitor LY294002 stabilised ERα protein under both untreated and TNFα-treated conditions. Similar results were obtained in ERα-8 cells. Increase in ERα protein in LY294002-treated cells was not due to increased transcription of ERα in LY294002-treated cells (Figure 4B). To further confirm the role of PI3-kinase on ERα stability, we examined the effect of LY294002 on TNFα-induced degradation of ERα in MCF-7 cells. Although LY294002 reduced the basal ERα protein level possibly due to its effects on general transcription, it blocked TNFα-induced but not E2-induced degradation of ERα (Figure 4C). Recent studies have...
shown that LY294002 inhibits both PI3-kinase and casein kinase II (Davies et al., 2000). We used apigenin, a casein kinase II inhibitor (Crichtfield et al., 1997), to support our conclusion that PI3-kinase is involved in TNFα-induced degradation of ERα. Apigenin failed to inhibit TNFα-induced degradation of ERα in MCF-7 cells (Figure 4D). In fact, apigenin on its own reduced ERα level. To further confirm the role of PI3-kinase in destabilisation of ERα, we transfected MCF-7 cells with constitutively active PI3-kinase and measured ERα protein 48 h after transfection. Oestrogen receptor alpha protein levels were lower in cells transfected with PI3-kinase expression vector (Figure 4E). Similar results were obtained in 293 and MDA-MB-231 cells transfected with ERα and constitutively active PI3-kinase (data not shown). Activation of AKT alone is sufficient for PI3-kinase-mediated destabilisation of ERα as a constituatively active AKT (CA-AKT) but not kinase-dead AKT (KD-AKT) reduced ERα levels (Figure 4E). It is possible that PI3-kinase-mediated destabilisation of ERα involves AKT-dependent phosphorylation of ERα followed by activation-coupled degradation. Our repeated attempts to establish MDA-MB-231 cells overexpressing ERα mutants that cannot be phosphorylated by cyclin A/cdk2 (S102N,104P,106A), MAPK/cdk7 (S118A), AKT/RSK (S167A), PKA (S236A) or SRC (Y537F) were unsuccessful. In transient transfection assays, phosphorylation-defective mutants were always expressed at a higher level than wild-type ERα (although expressed from a same promoter), suggesting that phosphorylation-defective mutants are more stable than wild-type ERα (data not shown).

Prolonged exposure of ERα-overexpressing cells to TNFα leads to loss of transrepression, which can be reversed partially by LY294002

To determine the consequences of stabilisation of ERα by LY294002 on transrepression, we treated parental and ERα-overexpressing cells with TNFα for 16 h or 3 days and measured IL-6 expression levels. Interleukin-6 expression in ERα-8 cells was lower than in parental cells after 16 h of TNFα treatment (Figure 5). However, IL-6 expression was similar in both LxSN23 and ERα-8 cells after 3 days of TNFα treatment. Interestingly, LY294002 was more effective in reducing TNFα-inducible IL-6 expression in ERα-8 cells compared to LxSN23 cells. Similar results were obtained in ERα-6 cells. Note that LY294002 had no effect on TNFα-inducible expression of TRAF-1. Thus, inhibition of TNFα-inducible IL-6 expression by LY294002 is less likely due to reduction in AKT/PKB-mediated activation of NF-κB or toxicity. We propose that LY294002 reduces TNFα-inducible IL-6 expression in ERα-overexpressing cells by enhancing transrepression function of ERα. In contrast to enhanced transrepression, LY294002 reduced transactivation by ERα (data not shown, Kishimoto and Nakshatri, submitted). Surprising specificity of LY294002 in inhibiting IL-6 but not TRAF-1 expression is encouraging as inhibitors with similar properties can be used to reduce invasion of breast cancer cells, more so of ERα-positive cancer cells, by specifically reducing IL-6 expression.

DISCUSSION

In this report, we show that the ERα protein level in breast cancer cells is regulated by TNFα and PI3-kinase, which has important implications on the transrepression function of ERα. Transrepression by ERα is believed to be responsible for reducing invasion and metastasis of ERα-positive breast cancers (Platel et al., 2000). Repression of gene expression appears to be a major function of ERα as recent studies show that among ~400 genes regulated by ERα in MCF-7 cells, ~70% of them are downregulated (Frasor et al., 2003). By lowering the ERα protein level, TNFα and PI3-kinase can overcome transrepression by ERα, thus promoting invasion and metastasis of breast cancers. Recent molecular profiling data with patient samples is consistent with the above observation. Patients with lower levels of ERα protein in their tumours have shorter disease-free survival rates than patients with higher levels of ERα in their tumours (Sorlie et al., 2001). It is interesting that PI3-kinase levels are higher in highly invasive and metastatic breast cancer cell line MDA-MB-231 cells compared to nonmetastatic MCF-7 cells (Sliva et al., 2002), which can explain for LY294002-induced stabilisation of ERα in MDA-MB-231 cells overexpressing ERα. Although our efforts to generate MCF-7 and MDA-MB-231 cells lacking PI3-kinase using siRNA technique were not successful, the failure of other kinase inhibitors including PD98059, PP2 and apigenin to alter the stability of ERα suggests that specific inhibition of PI3-kinase is responsible for LY294002-induced stabilisation of ERα. The PI3-kinase pathway appears to be involved in destabilisation of ERα by TNFα but not E2, as LY294002 inhibited TNFα-induced but not E2-induced degradation of ERα in MCF-7 cells (Figure 4). Our results differ to some extent from a recent report, which showed enhanced turnover of unliganded and liganded ERα in MCF-7 cells treated overnight with LY294002 (Marsaud et al., 2003). Authors suggested that PI3-kinase activity is required for stabilisation of ERα in MCF-7 cells. Some of the effect of LY294002 on ERα protein level in MCF-7 cells could be at the level of ERα transcription from the endogenous promoter as we also observed similar decrease in ERα protein in MCF-7 cells treated with LY294002. However, this is not the case when ERα is expressed through a heterologous promoter as LY294002 stabilised ERα in MDA-MB-231 derivatives ERα-6 and ERα-8 cells (Figure 4A). Several recent studies suggest that data generated using LY294002 alone should be interpreted cautiously. For example, LY294002 inhibits both PI3-kinase and casein kinase II at same concentration (Davies et al., 2000). LY294002 has been shown to bind to the LBD of ERα and inhibit its activity (Pasapera Limon et al., 2003). To ensure a direct role for PI3-kinase in destabilisation of ERα, we determined the effect of overexpression of constitutively active PI3-kinase on ERα protein levels in MCF-7 cells. PI3-kinase reduced ERα protein level in these cells (Figure 4E).
A major implication of this study is on sensitivity of breast cancer cells to chemotherapy. Interleukin-6 has been shown to increase motility and confer multidrug resistance to breast cancer cells (Tamm et al., 1991; Conze et al., 2001). By reducing basal and/or TNFα-induced IL-6 expression, ERα can reduce multidrug-resistant growth of breast cancer cells. Consistent with this possibility, in preliminary studies, we have observed increased sensitivity of ERα-overexpressing MDA-MB-231 cells to doxorubicin (data not shown). PI3-kinase inhibitors may further enhance the sensitivity of ERα-expressing cells to chemotherapy by stabilising ERα. We have recently observed inhibition of the transactivation function of ERα in tamoxifen-resistant breast cancer cells by PI3-kinase inhibitors (Kishimoto and Nakshatri, submitted). From these results, we propose that PI3-kinase inhibitors have the potential to overcome the multidrug resistance of ERα-positive breast cancers by simultaneously increasing transrepression and reducing transactivation by ERα.

How TNFα and PI3-kinase promote degradation of ERα remains to be determined. Although TNF-induced degradation of ERα has been reported, to our knowledge, this is the first report demonstrating a role for PI3-kinase in ERα degradation (Danforth and Spagias, 1993). Phosphatidylinositol-3-kinase has recently been shown to be required for degradation of β-arrestin-1 in response to chronic insulin treatment (Dalle et al., 2002). It is possible that TNFα and PI3-kinase induces the expression of a protein that targets ERα for proteosome-mediated degradation or it may induce the activity of proteosomal subunits, which alters the specificity of the proteosome. In this regard, it has been shown that TNFα increases ubiquitin-conjugating activity by increasing the expression of UbcH2 through NF-κB (Li et al., 2003). The other possibility is that TNFα alters the NEDD8 pathway, which has recently been shown to be involved in ERα degradation (Fan et al., 2003). Peroxisome proliferator-activated receptor gamma (PPARγ) and aryl hydrocarbon receptor also promote proteosomal degradation of ERα (Qin et al., 2003; Wormke et al., 2003). It is possible that TNFα utilises these receptors to promote ERα degradation. In this regard, kinetics and degree of TNF and aryl hydrocarbon receptor-induced degradation of ERα are similar. The other possibility is that TNFα, like IFNα, induces the replacement of proteosomal subunits, resulting in altered proteolytic specificity (Hishamatsu et al., 1996).

Although interaction between NF-κB and ERα was reported about 8 years ago (Stein and Yang, 1995), how that interaction leads to either transactivation or transrepression is not known. Initial studies suggested that competition for limiting coactivators is responsible for transrepression. However, subsequent studies by Parker’s group and this study with a mutant ERα rule out coactivator competition as being the primary mechanism of transrepression, at least in breast cancer cells (Sheppard et al., 1999; Harnish et al., 2000; Valentine et al., 2000). Also, our studies show that not all NF-κB-regulated genes are transrepressed by ERα, which suggests that transrepression involves specific promoter context. There may be similarity in transrepression by GR and ERα. Expression of IL-8 upon TNFα stimulation involves NF-καB-dependent assembly of the transcription preinitiation complex followed by phosphorylation of the RNA polymerase II carboxyl terminal domain. Glucocorticoid receptor has been shown to interfere with phosphorylation of the RNA polymerase II carboxyl terminal domain without interfering with the preinitiation complex formation (Nissen and Yamamoto, 2000). Transrepression by GR in some instances involves corecruitment of the coactivator molecule GRIP1, and the coactivators SRC-1 and SRC-3 cannot substitute this function of GRIP1 (Rogatsky et al., 2002). Promoter specificity in ERα-mediated transrepression may also involve a similar mechanism.

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REFERENCES

Ali S, Coombs RC (2002) Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2: 101 – 112
Ali S, Metzger D, Bornert JM, Chambon P (1993) Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J 12: 1153 – 1160
Ali SH, O’Donnell AL, Balu D, Pohl MB, Seyler MJ, Mohamed S, Moussa S, Dandona P (2001) High levels of oestrogen receptor-alpha in tumorigenesis: inhibition of cell growth and angiogenic factors. Cell Prolif 34: 223 – 231
Blobel GA, Sieff CA, Orkin SH (1995) Ligand-dependent repression of the erythroid transcription factor gamma TATA-1 by the estrogen receptor. Mol Cell Biol 15: 3147 – 3153
Campbell RA, Bhat-Nakshatri P, Patel NM, Constantindou D, Ali S, Nakshatri H (2001) PI3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 276: 9817 – 9824
Cerillo G, Rees A, Manchanda N, Reilly C, Brogan I, White A, Needham M (1998) The oestrogen receptor regulates NF kappaB and AP-1 activity in a cell-specific manner. J Steroid Biochem Mol Biol 67: 79 – 88
Conze D, Weiss L, Regen FS, Bhushan A, Weaver D, Johnson P, Rincon M (2001) Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. Cancer Res 61: 8851 – 8858
Crichtfield JW, Coligan JE, Folks TM, Butler SA (1997) Casein kinase II is a selective target of HIV-1 transcriptional inhibitors. Proc Natl Acad Sci USA 94: 6101 – 6115
Dalle S, Imamura T, Rose DW, Worrall DS, Ugi S, Hupfeld CJ, Olefsky JM (2002) Insulin induces heterologous desensitization of G-protein-coupled receptor and insulin-like growth factor I signaling by down-regulation of beta-arrestin-1. Mol Cell Biol 22: 6272 – 6285
Dandona P (2001) High levels of oestrogen receptor A/B region. Transcriptional activation by ligand-dependent phosphorylation of the estrogen receptor alpha inhibits IL-1beta induction of gene expression in the mouse liver. Endocrinology 143: 2599 – 2570
Di Croce L, Okret S, Kersten S, Gustafsson JA, Parker M, Wahl W, Beato M (1999) Steroid and nuclear receptors. Villesfranche-sur-Mer, France, May 25 – 27, 1999. EMBO J 18: 6201 – 6210
Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Samudio I, Klade MP, Vyhlidal C, Safe S (1999) Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. J Biol Chem 274: 32099 – 32107
Evans MJ, Lai K, Shaw LJ, Harnish DC, Chadwick CC (2002) Estrogen receptor alpha inhibits IL-1beta induction of gene expression in the mouse liver. Endocrinology 143: June – June
Fan M, Bigby RM, Nephew KP (2003) The NEDD8 pathway is required for proteosome-mediated degradation of human estrogen receptor (ER)-alpha and essential for the antiproliferative activity of ICI 182,780 in EAlpha-positive breast cancer cells. Mol Endocrinol 17: 356 – 365
Filardo EJ, Quinn JA, Bland KI, Frackelton Jr AR (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14: 1649 – 1660

British Journal of Cancer (2004) 90(4), 853 – 859 © 2004 Cancer Research UK
Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet Jr RJ, Sledge Jr GW, Newton TR, Patel NM, Bhat-Nakshatri P, Stauss CR, Goulet Jr RJ, Nakshatri, Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Metivier R, Stark A, Flouriot G, Hubner MR, Brand H, Penot G, Manu D, Marsaud V, Gougelet A, Maillard S, Renoir JM (2003) Various phosphor-

Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L (1996) Hisamatsu H, Shimbara N, Saito Y, Kristensen P, Hendil KB, Fujiwara T, Lonard DM, Nawaz Z, Smith CL, O’Malley BW (2000) The 26S protea-

Li YP, Lecker SH, Chen Y, Waddell ID, Goldberg AL, Reid MB (2003) TNF-

Le Goff P, Montano MM, Schodin DJ, Katzenellenbogen BS (1994)

Jilka RL, Hangoc G, Girasole G, Passeri G, Williams DC, Abrams JS, Boyce B, Broxmeyer H, Manolagas SC (1992) Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 257: 89 – 91

Lee H, Bai W (2002) Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol Cell Biol 22: 5835 – 5845

Le Goff P, Montano MM, Schodin DJ, Katzenellenbogen BS (1994) Phosphorylation of the human estrogen receptor. Identification of hormone-activated sites and examination of their influence on transcriptional activity. J Biol Chem 269: 4458 – 4466

Li YP, Lecker SH, Chen Y, Waddell ID, Goldberg AL, Reid MB (2003) TNF-

and expression. Mol Endocrinol 7: 50 – 56

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the second decade. Genes Dev 10: 939 – 948

Ma Z, Webb DJ, Jo M, Goniais SL (2001) Endogenously produced urokinase-

Li YP, Lecker SH, Chen Y, Waddell ID, Goldberg AL, Reid MB (2003) TNF-

Marsaud V, Gougelet A, Maillard S, Renoir JM (2003) Various phosphor-

Marsaud V, Gougelet A, Maillard S, Renoir JM (2003) Various phosphor-

Metsivuori J, Jussila L, Oinonen A, Nevalainen T, Kovanen PT (2000) The TAPA1/TAPA2 complex mediates TNF-alpha-induced migration of tumour cell lines. Mol Cell Biol 20: 5240 – 5249

Maurer H, Bhat-Nakshatri P, Martin DA, Goulet Jr RJ, Sledge Jr GW (1997) Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 17: 3629 – 3639

Neilson P, Patel NM, Bhat-Nakshatri P, Stuss C, Goulet Jr RJ, Nakshatri H (1999) Negative regulation of transcriptional function but not DNA binding of NF-kappaB and AP-1 by IkappaBalpha in breast cancer cells. J Biol Chem 274: 18827 – 18835

Nissen RM, Yamamoto KR (2000) The glucocorticoid receptor inhibits NF-kappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev 14: 2314 – 2329

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kusnjer PJ, Scanlan TS (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277: 1508 – 1510

Passapera Limon AM, Herrera-Munoz J, Gutierrez-Sagul R, Uloa-Aguirre A (2003) The phosphatidylinositol 3-kinase inhibitor LY294002 binds the estrogen receptor and inhibits 1beta-estradiol-induced transcriptional activity of an estrogen sensitive reporter gene. Mol Cell Endocrinol 200: 199 – 202

Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, Gelivanov F, Boswell SH, Goulet Jr RJ, Sledge Jr GW, Nakshatri H (2000) Paclitaxel sensitivity of breast cancer cells with constitutively active NF-

Patten K, Kuiper GG, Nilsson S, Gustafsson J, Kusnjer PJ, Scanlan TS (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277: 1508 – 1510

Plaet D, Cunat S, Chalbos D, Rochefort H, Garcia M (2000) Unliganded and liganded estrogen receptors protect against cancer invasion via different mechanisms. Mol Endocrinol 14: 999 – 1009.

Petratze ST, Bellido T, Mocheria H, Crabbe D, Manolagas SC (1994) 17 beta-

Estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. J Clin Invest 93: 944 – 950

Qin C, Burghardt R, Smith R, Worrnke M, Stewart J, Safe S (2003) Peroxosimone proliferator-activated receptor gamma agonists induce proteasome-dependent degradation of cyclin D1 and estrogen receptor alpha in MCF-7 breast cancer cells. Cancer Res 63: 958 – 964

Razandi M, Alton G, Pedram A, Ghoshani S, Webb P, Levin ER (2003) Identification of a structural determinant necessary for the localization and function of estrogen receptor alpha at the plasma membrane. Mol Cell Biol 23: 1633 – 1646

Rogatsky I, Luecke HF, Leitman DC, Yamamoto KR (2002) Alternate surfaces of transcriptional coregulator GRIP1 function in different estrogen receptor activation and repression contexts. Proc Natl Acad Sci USA 99: 16701 – 16706

Roth M, Wong SC, Henzel WJ, Goeddel DV (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 78: 681 – 692

Schroder J, Kahike V, Staubach KH, Zabel P, Stuber F (1998) Gender differences in human sperm. Arch Surg 133: 1200 – 1205

Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T (1999) Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol 19: 6367 – 6378

Simionetti C, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407: 538 – 541

Sliva D, Rizzo MT, English D (2002) Phosphatidylinositol 3-kinase and NF-

kappaB regulate motility of invasive MDA-MB-231 human breast cancer cells by the secretion of urokinase-type plasminogen activator. J Biol Chem 277: 3310 – 3317

Solvie T, Perou CM, Tishbihrani A, Raa T, Geisler S, Johnsen H, Haste T, Eiisen MB, van de Rijm J, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 98: 10869 – 10874

Stein B, Yang MX (1995) Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappaB and C/EBP beta. Mol Cell Biol 15: 4971 – 4979

Tamim I, Cardinale I, Murphy JS (1991) Decreased adherence of interleukin-6-treated breast carcinoma cells can lead to separation from neighbors after mitosis. Proc Natl Acad Sci USA 88: 4414 – 4418

Valentine JR, Kalkhoven E, White B, Hoare S, Parke MG (2000) Mutations in the estrogen receptor ligand binding domain discriminate between hormone-dependent transactivation and transpression. J Biol Chem 275: 25322 – 25329

Wang RA, Mazumdar A, Vadlamudi RK, Kumar R (2002) P21-activated kinase 1 phospholipase and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. EMBO J 21: 5437 – 5447

Worrnke M, Stoner M, Saville B, Walker K, Abdelrahim M, Burghardt R, Safe S (2003) The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes. Mol Cell Biol 23: 1843 – 1855

British Journal of Cancer (2004) 90(4), 853 – 859