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Adaptive hypersensitivity to estrogen: mechanism for superiority of aromatase inhibitors over selective estrogen receptor modulators for breast cancer treatment and prevention

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Abstract

Clinical observations suggest that human breast tumors can adapt to endocrine therapy by developing hypersensitivity to estradiol (E_2). To understand the mechanisms responsible, we examined estrogenic stimulation of cell proliferation in a model system and provided *in vitro* and *in vivo* evidence that long-term E_2 deprivation (LTED) causes 'adaptive hypersensitivity'. The enhanced responses to E_2 do not involve mechanisms acting at the level of transcription of estrogen-regulated genes. We found no evidence of hypersensitivity when examining the effects of E_2 on regulation of c-myc, pS2, progesterone receptor, several estrogen receptor (ER) reporter genes, or c-myc in hypersensitive cells. Estrogen deprivation of breast cells long-term does up-regulate both the MAP kinase and phosphatidylinositol 3-kinase pathways. As a potential explanation for up-regulation of these signaling pathways, we found that ER α is 4- to 10-fold up-regulated and co-opts a classic growth factor pathway using Shc, Grb-2 and Sos. This induces rapid non-genomic effects which are enhanced in LTED cells. E_2 binds to cell membrane-associated ER α , physically associates with the adapter protein SHC, and induces its phosphorylation. In turn, Shc binds Grb-2 and Sos, which results in the rapid activation of MAP kinase. These non-genomic effects of E_2 produce biological effects as evidenced by Elk activation and by morphological changes in cell membranes. Further proof of the non-genomic effects of E_2 involved use of cells which selectively expressed ER α in the nucleus, cytosol and cell membrane. We created these COS-1 'designer cells' by transfecting ER α lacking a nuclear localization signal and containing a membrane localizing signal.

The concept of 'adaptive hypersensitivity' and the mechanisms responsible for this phenomenon have important clinical implications. Adaptive hypersensitivity would explain the superiority of aromatase inhibitors over the selective ER modulators (SERMs) for treatment of breast cancer. The development of highly potent third-generation aromatase inhibitors allows reduction of breast tissue E_2 to very low levels and circumvents the enhanced sensitivity of these cells to the proliferative effects of E_2 . Clinical trials in the adjuvant, neoadjuvant and advanced disease settings demonstrate the greater clinical efficacy of the aromatase inhibitors over the SERMs. More recent observations indicate that the aromatase inhibitors are superior for the prevention of breast cancer as well. These observations may be explained by the hypothesis that estrogens induce breast cancer both by stimulating cell proliferation and by their metabolism to genotoxic products. The SERMs block ER-mediated proliferation only, whereas the aromatase inhibitors exert dual effects on proliferation and genotoxic metabolite formation.

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Introduction

Women with hormone-dependent breast cancer respond to therapeutic agents which either inhibit the synthesis or block the action of estrogens (Santen 2002). In order to compare the relative efficacy of these two strategies, clinical studies randomized women to receive either an aromatase inhibitor to block estrogen synthesis, or the antiestrogen tamoxifen. These clinical trials demonstrated the superiority of aromatase inhibitors in the neoadjuvant, adjuvant and advanced disease settings. In addition, preliminary data suggest the superiority of the aromatase inhibitors for prevention of contralateral breast cancer during adjuvant therapy (ATAC Trialists' Group 2002, Buzdar 2002). These unexpected observations suggest that the antiestrogens and aromatase inhibitors exert inherently different effects mechanistically. A variety of recent studies provide molecular and tumor biological data to identify these mechanisms and to explain the superiority of aromatase inhibitors over the antiestrogens. This manuscript will review our work and that of others which provides a mechanistic framework to differentiate the effects of the aromatase inhibitors from those of the antiestrogens.

Historical aspects

Clinical observations from two decades ago provided clues regarding the differential effects of aromatase inhibitors and antiestrogens. One would have expected complete cross-resistance between aromatase inhibitors and tamoxifen because both provide a means to block the cellular effects of estrogen. However, this was not the case. Fifty percent of women responding initially to tamoxifen but then relapsing, experienced secondary clinical benefit when crossed over to aminoglutethimide, a first-generation aromatase inhibitor (Santen *et al.* 1990). This observation provided evidence that aminoglutethimide would be efficacious as second-line therapy for women with hormone-dependent breast cancer and that different mechanisms of action must be operative with respect to aromatase inhibitors and tamoxifen.

Adaptive hypersensitivity

To explain the lack of complete cross-resistance between aromatase inhibitors and antiestrogens, we and other investigators postulated the phenomenon of 'adaptive hypersensitivity' (Santen 1996, Masamura *et al.* 1995, Costa *et al.* 1999, Shim *et al.* 2000, Jeng *et al.* 2000, Song 2002a, 2002b). This hypothesis suggested that tamoxifen might exert pressure causing breast cancer cells to adapt and develop hypersensitivity to the estrogenic properties of tamoxifen (Santen 1996). It is well known that tamoxifen, as a representative of the class of drugs called selective estrogen receptor (ER)

modulators or SERMs, exerts both estrogen agonistic or antagonistic properties depending upon the tissue examined and circumstances involved (Jordan *et al.* 1997). From the concept of tamoxifen-induced hypersensitivity, one could develop a strategy applicable to treatment of women with breast cancer. Circumvention of hypersensitivity would involve cessation of tamoxifen and use of a potent aromatase inhibitor to reduce estradiol (E_2) to very low levels. With this as a rationale, one would expect secondary tumor regressions, and clinical observations demonstrated this phenomenon when using second-line aromatase inhibitors (Santen *et al.* 1990).

Hormone-ablative therapy involving deprivation of estrogen production surgically might also induce hypersensitivity. Clinical observations in pre-menopausal women with breast cancer supported that possibility (Santen *et al.* 1990). Hormone-dependent breast cancers often regress in response to surgical removal of the ovaries, a treatment which lowers circulating plasma E_2 from approximately 200 to 15 pg/ml (Santen & Harvey 1999). In response to this acute deprivation of E_2 , tumors regress for 12–18 months on average before they begin to regrow. Second-line therapy with surgical oophorectomy or with aromatase inhibitors can then induce additional tumor regressions by lowering E_2 concentrations further to 1–5 pg/ml (Santen *et al.* 1990). These observations first demonstrated enhanced sensitivity to circulating E_2 . Specifically, 200 pg/ml E_2 were required to stimulate tumor growth before oophorectomy whereas levels of 15 pg/ml were sufficient to cause tumor proliferation after adaptation 12–18 months later.

We and other investigators sought to directly demonstrate the phenomenon of adaptive hypersensitivity and to determine the mechanisms involved. We utilized a model system involving MCF-7 human breast cancer cells *in vitro* (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Herman & Katzenellenbogen 1994, Jeng *et al.* 1998, 2000, Shim *et al.* 2000). Wild-type MCF-7 cells are cultured over a prolonged period in estrogen-free medium to mimic the effects of ablative endocrine therapy such as induced by surgical oophorectomy (Masamura *et al.* 1995). This process involves long-term E_2 deprivation; the adapted cells are called by the acronym LTED cells. In response to E_2 deprivation, MCF-7 cells initially stop growing but then, 3–6 months later, adapt and grow as rapidly as wild-type MCF-7 cells maximally stimulated with E_2 . We attribute this effect to the development of hypersensitivity to E_2 with re-growth in response to residual amounts of estrogen in the charcoal-stripped culture media. Recent data from our laboratory (W Yue, J-P Wang & R J Santen unpublished observations) and that of Ishikawa *et al.* (2001) demonstrated that the residual E_2 present is leached from plastic culture plates and results from plasticizers such as nonyl-phenol derivatives.

We have provided direct evidence of hypersensitivity by showing that a four-log lower concentration of E_2 can stimu-

late proliferation of LTED cells compared with wild-type MCF-7 cells (Fig. 1) (Masamura *et al.* 1995). *In vivo* studies also demonstrated that LTED cell xenografts grown in nude mice are hypersensitive to low doses of E_2 . A key question is whether long-term exposure to tamoxifen similarly induces a state of estrogen hypersensitivity. Studies by Osborne & Fuqua (1994) and by Jordan and colleagues (Gottardis *et al.* 1989) demonstrated that long-term exposure to tamoxifen in nude mice caused MCF-7 cells to adapt and to respond to the estrogen agonistic properties of tamoxifen. (The stimulatory effects of tamoxifen under these circumstances could be blocked by the pure antiestrogen, fulvestrant.)

We (Bernstein *et al.* 2003) have recently demonstrated that long-term exposure of MCF-7 xenografts to tamoxifen in castrated nude mice also induces a state of hypersensitivity to E_2 (Fig. 2). During the phase when tamoxifen first begins to stimulate tumor growth, removal of tamoxifen and administration of very low doses of E_2 stimulates tumor growth. In marked contrast, MCF-7 xenografts receiving vehicle only are not stimulated by such low doses of E_2 . Taken together, these experiments indicate that either E_2 deprivation or blockade of estrogen action with antiestrogens enhances the level of sensitivity to estrogens or to the estrogenic properties of tamoxifen. We postulate that this phenomenon of adaptive hypersensitivity explains the superiority of aromatase inhibitors over tamoxifen in a variety of clinical settings.

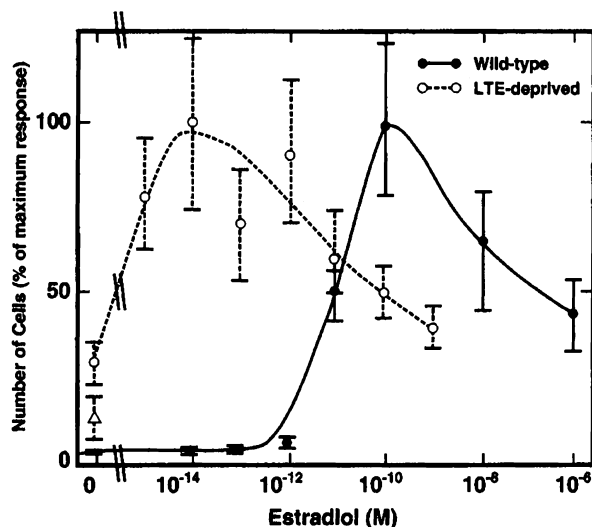


Figure 1 E_2 stimulation of growth of wild-type MCF-7 and LTED cells. E_2 dose–response curve in LTED (LTE-deprived) cells grown in serum-free media and wild-type cells grown in standard charcoal-stripped media. Data are illustrated as a percentage of the maximum response (\pm S.E.M.) to normalize data between experiments. ● Wild-type MCF-7 cells; ○ LTED cells; △ LTED cells treated with ICI 164384. Reprinted from Masamura *et al.* (1995) with kind permission of the publisher.

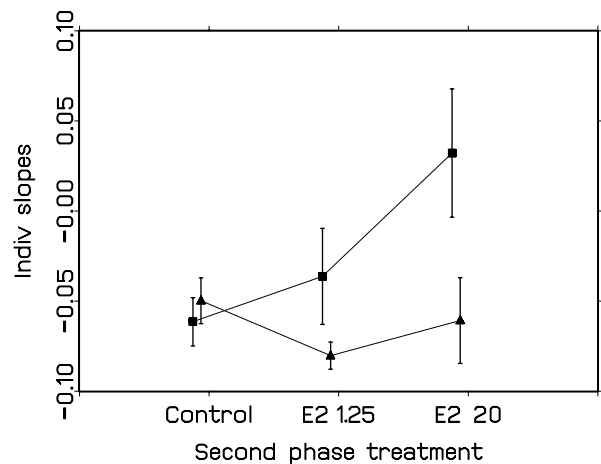


Figure 2 MCF-7 cells were implanted in castrated nude mice and exposed to tamoxifen or vehicle for a period of 5 months. The tamoxifen was then removed and animals were treated either with vehicle or with silastic E_2 implants to produce plasma E_2 levels of 1.25 and 20 pg/ml. The data points and error bars represent the slopes of growth curves over a 6 week period (Bernstein *et al.* 2003). ■–■, tamoxifen pre-treated; ▲–▲, vehicle pre-treated.

Mechanisms for adaptive hypersensitivity

This process could involve modulation of the genomic effects of E_2 acting on transcription, non-genomic actions involving plasma membrane related receptors, cross-talk between growth factor and steroid hormone-stimulated pathways, or interactions among these various effects (Jeng *et al.* 2000). We initially postulated that enhanced receptor-mediated transcription of genes related to cell proliferation might be involved. Indeed, the levels of $ER\alpha$ increased 4- to 10-fold during LTED. Accordingly, to directly examine whether enhanced sensitivity to E_2 in LTED cells occurred at the level of ER-mediated transcription, we quantitated the effects of E_2 on transcription in LTED and in wild-type MCF-7 cells. As transcriptional readouts, we measured the effect of E_2 on c-myc message levels, progesterone receptor (PgR) and pS2 concentrations, and on ERE-CAT reporter activity (Fig. 3) (Yue & Wang 2002). Although basal levels of pS2 and PgR were increased, we observed no shift to the left in E_2 dose–response curves (the endpoint utilized to detect hypersensitivity) for any of these responses when comparing LTED with wild-type MCF-7 cells. These data suggest that hypersensitivity of LTED cells to E_2 does not occur at the level of ER-mediated gene transcription.

We next considered that adaptation might involve dynamic interactions between pathways utilizing steroid hormones and growth factors for signaling. Both E_2 and various peptide growth factors are mitogens for breast tissues (Osborne *et al.* 1980, Stampfer *et al.* 1980, Dickson *et al.* 1986, Clarke *et al.* 1989). A variety of studies indicate that growth factor secretion and action can be stimulated by E_2

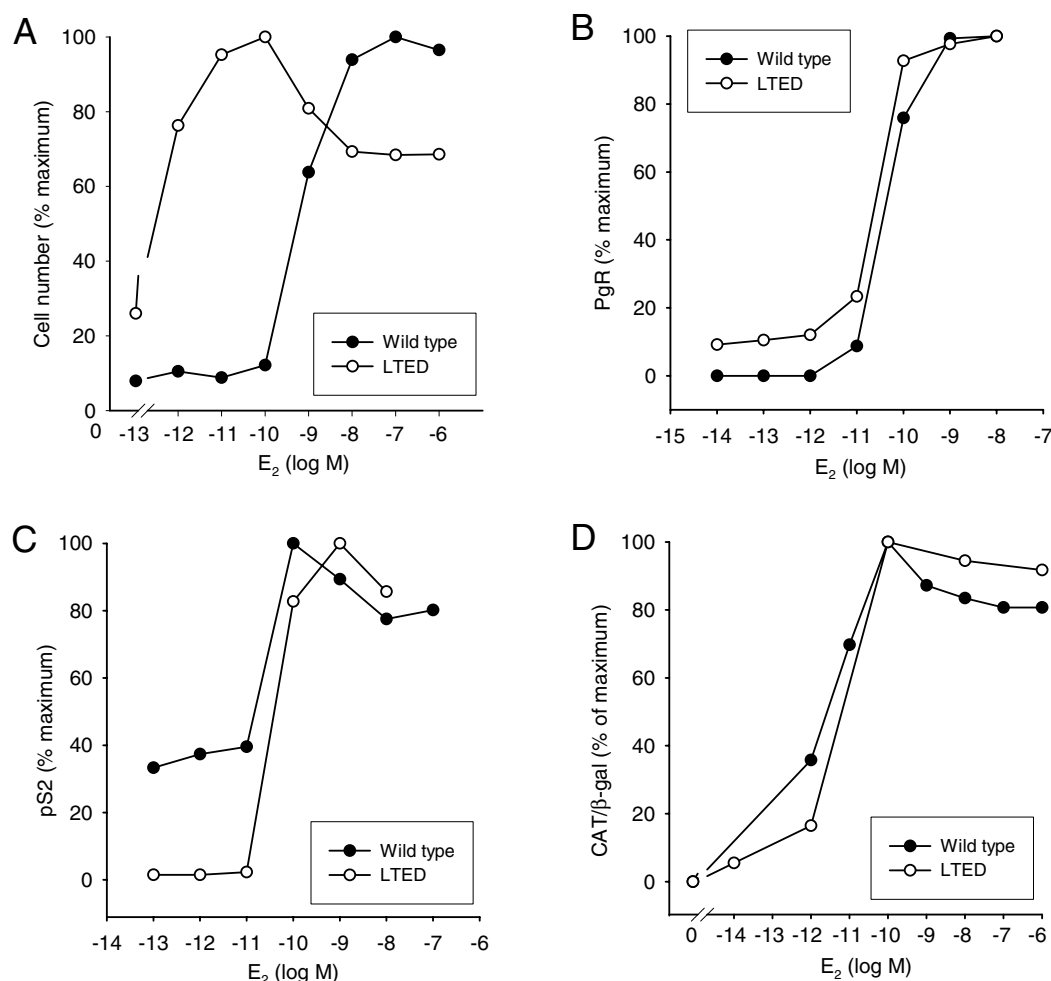


Figure 3 E_2 -induced cell proliferation, expression of PgR and pS2 proteins and ERE-CAT reporter activity. Wild-type MCF-7 and LTED cells were plated in six-well plates at a density of 60×10^3 cells/well in corresponding medium. After 2 days, the cells were re-fed with phenol red- and serum-free IMEM and cultured in this medium for another 2 days before treatment with various concentrations of E_2 in the presence of ICI 182780 (ICI) (10^{-9} M). Cell number was counted 5 days after treatment. (A) Percent of maximum cell number in wild-type MCF-7 and LTED cells treated with different concentrations of E_2 , (B) cytosol PgR under similar conditions, (C) pS2 protein under similar conditions and (D) ERE-CAT activity were measured 48 h after E_2 treatment. Reprinted from Yue & Wang (2002) in *Endocrinology* with the permission of the authors and publisher.

(Dickson *et al.* 1986, Liu *et al.* 1987). These effects are believed to result from the genomic effects of E_2 to stimulate transcription of early response genes such as c-myc and growth factors such as transforming growth factor (TGF)- α (Nass & Dickson 1997, Clarke *et al.* 1989, Dickson & Lippman 2001). Growth factors result in MAP kinase activation, which directly and indirectly enhances the degree of phosphorylation of the ER. MAP kinase directly phosphorylates serine 118 (Kato *et al.* 2000) and also stimulates Elk and RSK activity, which phosphorylate serine 167 (Joel *et al.* 1998).

Our initial approach determined if basal levels of MAP kinase were elevated in LTED cells. We demonstrated this

directly by measuring the level of activated MAP kinase in LTED cells *in vitro* (Fig. 4A) and in LTED xenografts in nude mice (Jeng *et al.* 2000, Shim *et al.* 2000). We further demonstrated that activated MAP kinase is implicated in the enhanced growth of LTED cells since inhibitors of MAP kinase such as PD98059 or U-0126 block the incorporation of tritiated thymidine into DNA (Jeng *et al.* 1998, Yue & Wang 2002). Upstream inhibitors of the MAP kinase pathway such as mevastatin or genestein, also block tritiated thymidine uptake. These data suggest that an increase in activated MAP kinase participates in the adaptive hypersensitivity process.

To demonstrate proof of the principle of MAP kinase participation, we stimulated activation of MAP kinase in

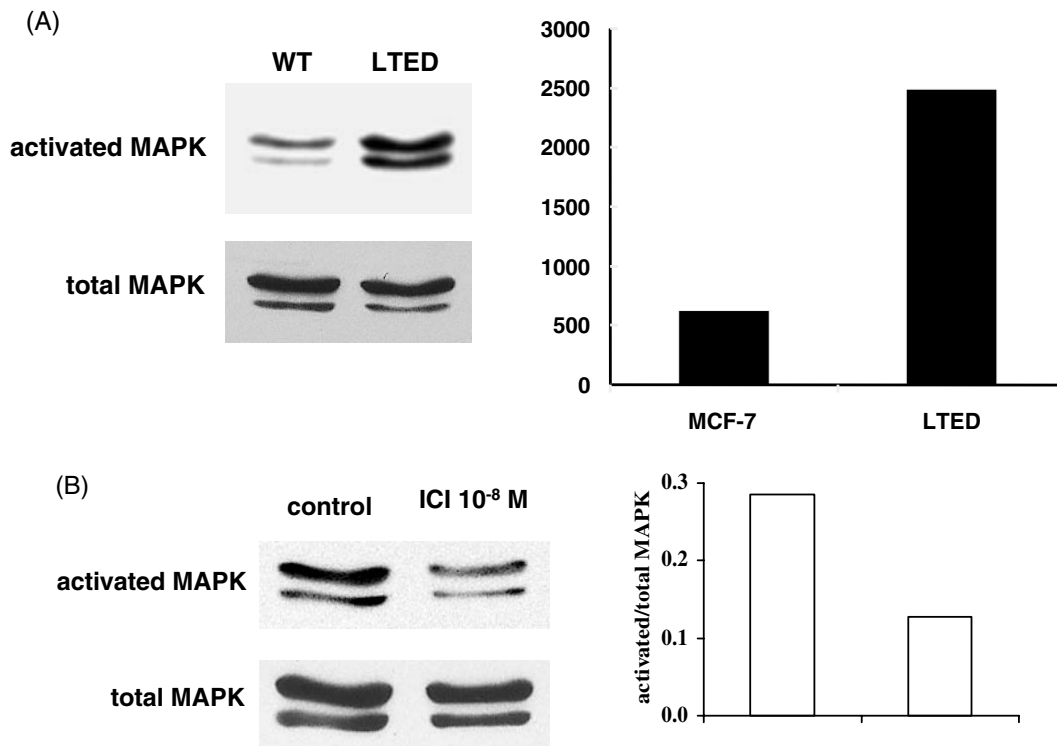


Figure 4 (A) Basal levels of activated and total MAP kinase (MAPK) in wild type (WT) and LTED cells. The western blots are shown on the left portion of the panel and the scanning densitometry data on the right (Yue *et al.*). (B) Similar representation of activated and total MAP kinase in LTED cells under control conditions and when exposed to ICI at a concentration of 10^{-8} M (Yue *et al.* 2002 reprinted from *Endocrinology* with kind permission from the publisher). The western blots are shown on the left portion of the panel and the scanning densitometry data on the right.

wild-type MCF-7 cells by administering TGF- α (Fig. 5A and B). Initial characterization data demonstrated increases in MAP kinase in MCF-7 cells with doses of TGF- α ranging from 0.1 to 10 ng/ml and blockade of this effect with the MAP kinase inhibitor PD98059 (Yue & Wang 2002). Administration of TGF- α at a dose of 10 ng/ml caused a two-log shift to the left in the ability of E_2 to stimulate the growth of wild-type MCF-7 cells. To demonstrate that this effect related specifically to MAP kinase and not to a non-MAP kinase-mediated effect of TGF- α , we co-administered PD98059. Under these circumstances, the two-log left shift in E_2 dose-response returned back to the baseline dose-response curve (Fig. 5B). As further evidence of the role of MAP kinase, we administered U-0126 to LTED cells and examined its effect on the level of sensitivity to E_2 . This agent partially shifted dose-response curves to the right by approximately one-half log. Taken together, these data suggest that MAP kinase activation does participate mechanistically in the adaptive hypersensitivity process.

While an important component, MAP kinase did not appear to be solely responsible for hypersensitivity to E_2 . Blockade of this enzyme did not completely abrogate hypersensitivity. Accordingly, we examined the phosphatidylinositol 3 kinase (PI-3 kinase) pathway to determine if it was

up-regulated in LTED cells as well. In preliminary experiments, we determined that LTED cells exhibit an enhanced activation of AKT, P70 S6 kinase, and 4EBP-1 (all components of the PI-3 kinase pathway) (Yue 2003b). Dual inhibition of PI-3 kinase with Ly 294002 (specific PI-3 kinase inhibitor) and MAP kinase with U-0126 shifted the level of sensitivity to E_2 more dramatically: more than two logs to the right. While we are currently pursuing these observations in more detail, our working hypothesis is that adaptive hypersensitivity involves the joint activation of the PI-3 kinase and MAP kinase pathways.

Additional studies examined why activated MAP kinase levels might be elevated in LTED cells. Up-regulation of MAP kinase could reflect either a constitutive activation of growth factor receptors, an increase in the endogenous secretion of growth factors, or other mechanisms. We reasoned that inhibition of MAP kinase with a pure antiestrogen would rule out the possibility of constitutive activation of growth factor receptors or growth factor secretion. Accordingly, we administered the pure antiestrogen, Faslodex (fulvestrant) and examined the level of activation of MAP kinase in LTED cells. Surprisingly, fulvestrant returned the level of activated MAP kinase back to the level seen in wild-type MCF-7 cells (Fig. 5B). This observation ruled out

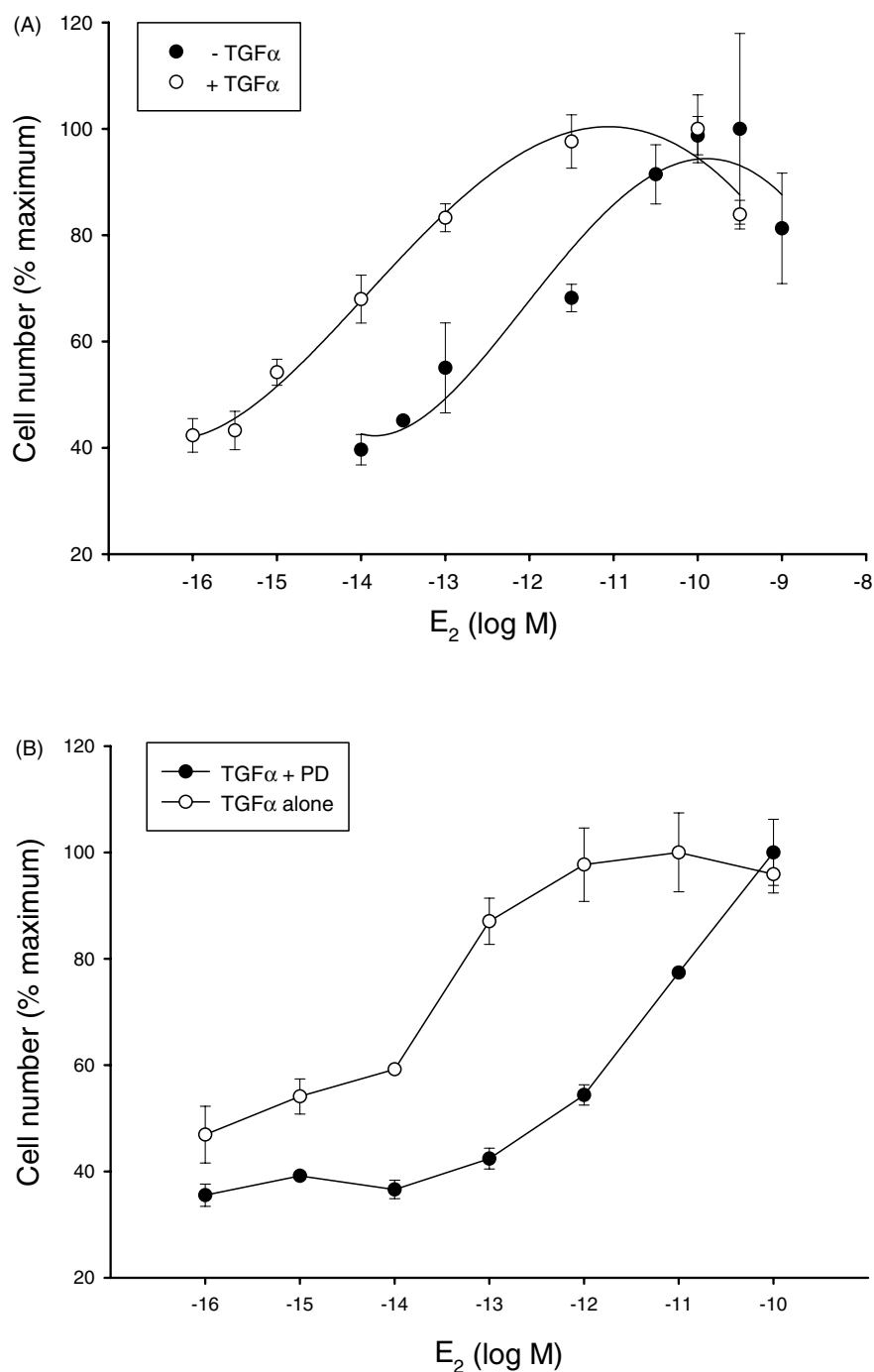


Figure 5 (A) TGF- α enhanced E_2 sensitivity in wild-type MCF-7 cells; 60×10^3 cells per well were plated in six-well plates and allowed to grow for 2 days in a medium containing phenol red and 5% fetal bovine serum. The cells were then re-fed with phenol red-free and serum-free medium. Two days later, the cells were treated with different concentrations of E_2 in the presence or absence of 10 ng/ml TGF- α in triplicate wells. Five days later, cell numbers were counted. Data from seven repeated experiments were used for statistical analysis and calculation of the EC50. The figure shows data from one representative experiment, expressed as mean cell numbers \pm S.E.M. (Yue *et al.* 2002). (B) PD98059 (PD) reversed TGF- α -enhanced E_2 sensitivity in wild-type MCF-7 cells. The experimental conditions are similar to those in (A). Data from two experiments were used for statistical analysis and calculation of the EC50. The figure shows the data from one experiment expressed as mean cell numbers \pm S.E.M. (Yue *et al.* 2002, reprinted from *Endocrinology* with kind permission of the publisher).

constitutive growth factor effects in LTED cells and suggested an interaction between ER-mediated functions and the elevation of MAP kinase.

Non-genomic membrane ER actions

One possible mechanism to explain the activation of MAP kinase would be through non-genomic effects of estrogen acting through ER α located in or near the cell membrane. Non-genomic actions of E₂ have only been recognized recently and encompass activation of MAP kinase, Ras, Raf-1, PKC, PKA and Maxi-K channels, elevation of intracellular calcium levels and release of nitric oxide (Migliaccio *et al.* 1996, 1998, Kelly *et al.* 1999, Valverde *et al.* 1999, Stefano *et al.* 2000). We postulated that membrane-associated ER α might utilize a classic growth factor pathway to transduce its effects in LTED cells (Fig. 6). The adaptor protein Shc represents a key modulator of tyrosine kinase-activated peptide hormone receptors. As an upstream regulator of MAP kinase, Shc transduces mitogenic

and differentiation signals from a variety of tyrosine kinase receptors such as epidermal growth factor receptor, nerve growth factor receptor, platelet-derived growth factor receptor and insulin-like growth factor receptor, to downstream kinase cascades (Dikic *et al.* 1995, Pelicci *et al.* 1995, 1996). Upon receptor activation and auto-phosphorylation, Shc binds rapidly to specific phosphotyrosine residues of receptors through its PTB or SH2 domain and becomes phosphorylated itself on tyrosine residues of the CH domain (Pelicci *et al.* 1995, 1996). The phosphorylated tyrosine residues on the CH domain provide the docking sites for the binding of the SH2 domain of Grb2 and hence recruit Sos, a guanine nucleotide exchange protein. Formation of this adapter complex allows Ras activation via Sos, leading to the activation of the MAP kinase pathway (Blenis 1993, Pelicci *et al.* 1995, 1996, Boney *et al.* 2000).

We postulated that estrogen deprivation might trigger activation of a non-genomic, estrogen-regulated, MAP kinase pathway which utilizes Shc (Song *et al.* 2002a, b). We

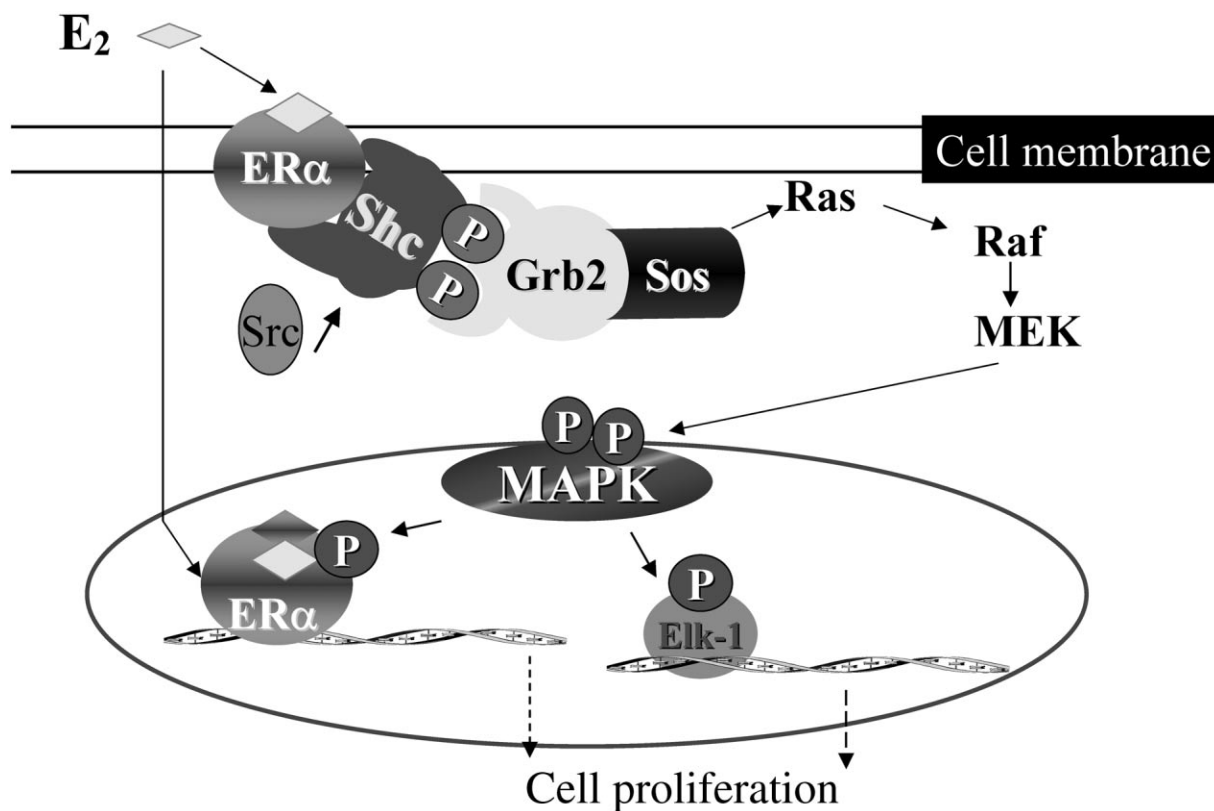


Figure 6 Diagrammatic representation of the two pathways involved in estrogen action. The genomic pathway involves the entry of E₂ into the cell and migration to the nucleus where E₂ binds to the ER and initiates gene transcription. Via the non-genomic pathway, E₂ binds to an ER near or in the cell membrane. There it initiates binding of Shc to ER α , phosphorylation of Shc through action of the kinase, c-Src, and then association with Grb-2 and Sos. Later events include conversion of GDP-Ras to GTP-Ras, and then activation of MEK and MAPK. We postulate that these two actions, genomic and non-genomic, are responsible for the growth of LTED cells and that activation of the non-genomic pathway is involved in induction of hypersensitivity of LTED cells.

employed MAP kinase activation as an endpoint with which to demonstrate rapid non-genomic effects of E_2 . The addition of E_2 stimulated MAP kinase phosphorylation in LTED cells within minutes. The increased MAP kinase phosphorylation by E_2 was time- and dose-dependent, being greatly stimulated at 15 min and remaining elevated for at least 30 min. Maximal stimulation of MAP kinase phosphorylation was at 10^{-10} M E_2 .

We then examined the role of peptides known to be involved in growth factor signaling pathways that activate MAP kinase. Shc proteins are known to couple tyrosine kinase receptors to the MAP kinase pathway and activation of Shc involves the phosphorylation of SHC itself (Dikic *et al.* 1995). To investigate if the Shc pathway was involved in the rapid action of E_2 in LTED cells, we immunoprecipitated tyrosine phosphorylated proteins and tested for the presence of Shc under E_2 treatment. E_2 rapidly stimulated Shc tyrosine phosphorylation in a dose- and time-dependent fashion with a peak at 3 min. The pure ER antagonist, fulvestrant, blocked E_2 -induced Shc and MAP kinase phosphorylation at 3 and 15 min respectively. The time frame suggests that Shc is an

upstream component in E_2 -induced MAP kinase activation (Song *et al.* 2002b).

To provide direct evidence of the necessity of Shc for MAP kinase activation, a glutathione S-transferase (GST)-tagged full-length Shc mutant (ShcFFF) with point mutations at tyrosines 239/240 and 317 (Y239/240/317F) was transfected into LTED cells (Song *et al.* 2002b). These three sites of tyrosine phosphorylation of Shc are important for its interaction with Grb2 and for transduction of the signal to downstream components. Expression of dominant negative ShcFFF markedly inhibited the ability of E_2 to stimulate MAP kinase phosphorylation (Fig. 7). This allowed us to conclude that Shc was necessary for activation of MAP kinase (Song *et al.* 2002b).

The adapter protein Shc may directly or indirectly associate with $ER\alpha$ in LTED cells and thereby mediate E_2 -induced activation of MAP kinase. We considered this likely in light of recent evidence regarding $ER\alpha$ membrane localization (Collins & Webb 1999, Watson *et al.* 1999a,b). To test this hypothesis, we immunoprecipitated Shc from non-stimulated and E_2 -stimulated LTED cells and then probed immunoblots

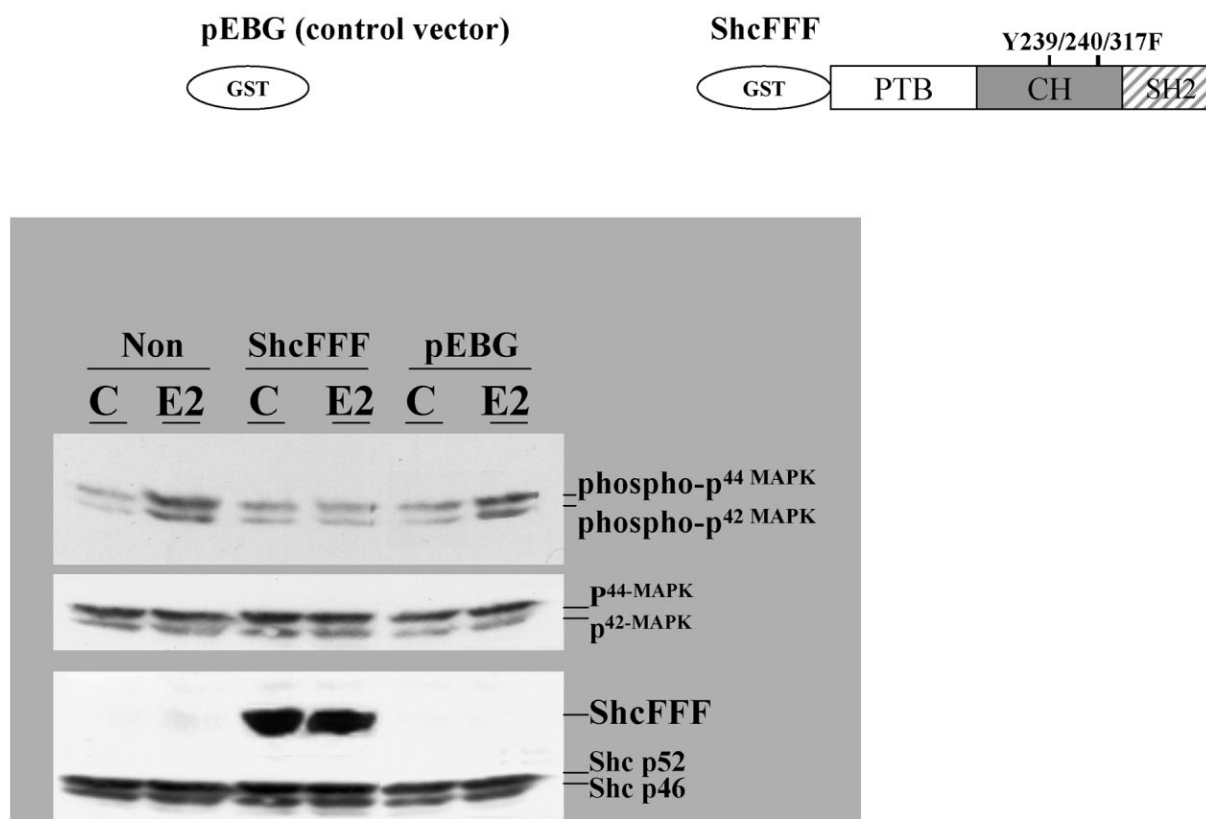


Figure 7 Overexpression of mutant Shc blocked E_2 -induced MAPK phosphorylation. MCF-7 cells were either not transfected or transfected with the mutant Shc (Shc FFF) or control vectors (pEBG) for 2 days. Then cells were treated with vehicle or 10^{-10} M E_2 for 15 min. Phosphorylated (top blot) and total MAPK (middle blot) were detected. Both GST-Shc-FFF and endogenous Shc expression are shown (bottom two blots). The above experiments have been repeated three times and one of the representative experiments is shown (Song *et al.* 2002b, reprinted from *Molecular Endocrinology* with kind permission of the publisher).

with anti-ER α antibodies. Our data showed that the ER α /Shc complex pre-existed before E₂ treatment and E₂ time-dependently increased this association (Song *et al.* 2002b). In parallel with Shc phosphorylation, we observed a maximally induced association between ER α and Shc at 3 min.

MAP kinase pathway activation by Shc requires Shc association with the adapter protein Grb2 and then further association with Sos. By immunoprecipitation of Grb2 and detection of both Shc and Sos, we demonstrated that the Shc–Grb2–Sos complex constitutively existed at relatively low levels in LTED cells, but was greatly increased by treatment of cells with 10^{–10} M E₂ for 3 min.

Because ER α , Shc and MAP kinase are all involved in E₂ action in MCF-7 cells, we wished to determine upstream components responsible for Shc phosphorylation. We suspected that c-Src might phosphorylate Shc in response to E₂ since Shc has been reported to be a substrate of Src tyrosine kinase in HEK-293 cells (Sato *et al.* 2000). On the other hand, we wished to demonstrate a role for ER α in this process and to exclude MAP kinase as the cause of Shc phosphorylation. Accordingly, we examined the effects of PP2 (an inhibitor of Src-family kinases), ICI 182780 (ICI) and PD98059 on E₂-induced phosphorylation of Shc. In the presence of the inhibitors, MCF-7 cells were stimulated with vehicle or 10^{–10} M E₂ for 3 min and the status of Shc phosphorylation was examined. Both PP2 and ICI effectively inhibited E₂-induced Shc phosphorylation, implying that Src family kinases and ER α are required for Shc activation. As expected, PD98059 did not influence the phosphorylation status of Shc, suggesting that it functions downstream of Shc. No effects of these inhibitors were apparent in the absence of E₂ stimulation. Taken together, these results indicate that both ER α and Src are upstream components of Shc functionality and their involvement is required for Shc phosphorylation. Each of these inhibitors was capable of reducing the rate of cell proliferation in LTED cells.

Biological effects of non-genomic pathway activation

To provide evidence that the ER α –Shc–MAP kinase pathway exerts biological effects, we evaluated the role of MAP kinase in the activation of Elk-1. When activated, Elk-1 serves as a downstream mediator of cell proliferation. The phosphorylation of Elk1 by MAP kinase can up-regulate its transcriptional activity through phosphorylation. By co-transfection of LTED cells with both GAL4-Elk and its reporter gene GAL4-luc (Roberson *et al.* 1995, Duan *et al.* 2001) we were able to show that E₂ dose-dependently increased Elk-1 activation at 6 h as shown by luciferase assay (Song *et al.* 2002b).

We also wished to demonstrate biological effects on cell morphology. It has been reported that cell mobility is controlled by a network of membrane-initiated signals, such as

activation of the Shc–Ras–MAP kinase pathway (Adam *et al.* 1998). Recently, Smilenov *et al.* (1999) reported that cell focal adhesions are highly dynamic structures. Cells can rapidly respond to the stimulation by growth factors and show reorganization of their cytoskeleton and cell shape. To examine E₂ effects on reorganization of the actin cytoskeleton, we visualized the distribution of F-actin by phalloidin staining and also redistribution of the ER α localization in LTED and MCF-7 cells (Fig. 8). Untreated LTED cells expressed low actin polymerization and a few focal adhesion points. After E₂ stimulation, in contrast, the cytoskeleton underwent remodeling associated with formation of cellular ruffles, lamellipodias and leading edges, alterations of cell shape and loss of mature focal adhesion points. A subcellular redistribution of ER α to these dynamic membranes upon E₂ stimulation represented another important feature of LTED cells. The ER antagonist ICI 182780 at 10^{–9} M blocked E₂-induced ruffle formation as well as redistribution of ER α to the membrane with little effect by itself. Therefore, these studies further demonstrated the rapid action of E₂ with respect to dynamic membrane alterations in LTED cells.

Use of confocal microscopy and immunofluorescence provided a dynamic means of assessing ER α location and alterations in response to E₂ (Fig. 8). Accordingly, we focused on the regions contiguous to the cell membrane. Under basal conditions, a faint green staining (i.e. ER immunofluorescence) could be observed along the cell membrane of the cells (Fig. 8). In marked contrast, E₂ appeared to translocate ER α into the region along the membrane ruffles as indicated by the strong appearance of green staining. As shown by merging the red (actin) and green (ER α) views, the ER α appeared as yellow (Fig. 8, insert b), indicating co-localization with actin in the membrane ruffles. Striking also was the translocation of the ER α into the ‘fist-like’ region of the pseudopodia as shown by both the green staining and yellow merged views. ICI blocked E₂-induced ER α membrane translocation but exerted little effect under basal conditions.

To provide further proof of non-genomic ER α -mediated effects, we constructed a series of designer ERs (Zhang *et al.* 2002). We obtained the ERs generated by the group of Dr Pierre Chambon which lacked a nuclear localization signal. To this, we coupled a 43 amino acid membrane localization sequence called Gap 43 to the ER. Gap 43 is used in the CNS to bring proteins to the membrane. We then transfected COS cells lacking an ER with the three ER α constructs. With dual fluorescence microscopy, we could demonstrate nearly exclusive localization of the wild-type ER α to the nucleus and of the ER lacking the nuclear localization signal to the cytoplasm. Receptor containing the membrane localization signal concentrated in the plasma membrane but also was found in the cytoplasm. Only the membrane ER α responded to exogenous E₂ with MAP kinase activation. In addition, only the membrane-localized ER α stimulated cell

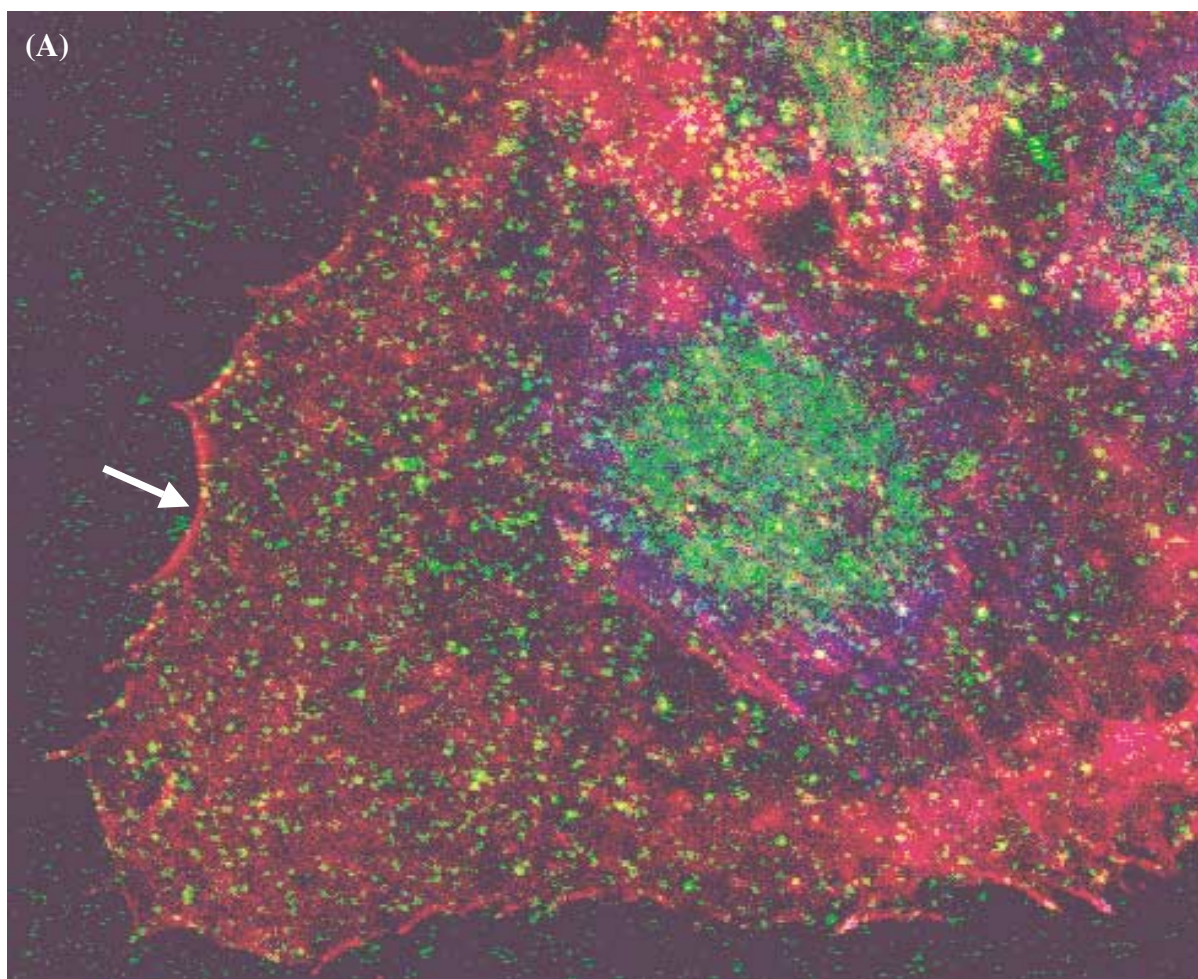


Figure 8 Confocal analysis of E_2 -induced morphological changes and $ER\alpha$ subcellular localization in LTED-MCF-7 cells. (A) Merged three color image of vinculin-stained untreated cells. Cells are characterized by low to moderate actin polymerization (red) with a few focal adhesion points (blue), and a significant nuclear $ER\alpha$ localization (green). A modest amount of green-color stained $ER\alpha$ is apparent in cytosol and in the peri-membrane area (see insert (a), for an expended view of the area marked by an arrow). (B and C) Cells were treated with 10^{-10} M E_2 for 20 min. Cells displayed formation of F-actin-containing dynamic membranes (red), such as ruffles (B) or pseudopodia (C). Membrane-bound $ER\alpha$ (green) appeared in the ruffles and peri-membrane ER in the pseudopodia. The co-localization of $ER\alpha$ with F-actin is visualized as yellow color as highlighted in the inserts (b) and (c) respectively, as a result of the co-localization of red and green pixels. This was accompanied by a total loss of the focal adhesion points (blue) and a dramatic change in the cell shape, with appearance of a leading edge (shown by an arrow in B). (D) cells were pretreated (10 min) with 10^{-9} M ICI 182780 and then treated with 10^{-10} M E_2 . ICI treatment significantly blocked the E_2 -induced ruffle formation (red) as well as redistribution of $ER\alpha$ to the membranes (green). Persistence of mature focal adhesion points (blue) indicates that the antiestrogen, ICI 182780, blocks the effects of E_2 observed in (B) and (C) (see also insert (d)). (E) cells were treated only with ICI. Low actin polymerization (red), peripherally disposed focal adhesion points (blue) and nuclear $ER\alpha$ localization characterize these cells. (See also insert (e)). (F) The inserts represent the details of the membranes for each treatment taken from areas highlighted by an arrow. Left panels represent the merged three colors, for F-actin (red), $ER\alpha$ (green) and vinculin (blue). The right panels show the $ER\alpha$ membrane localization and where present, the vinculin (blue). (a) This panel represents merged three-color images of vinculin, anti- $ER\alpha$ and actin-stained cells. These cells are characterized by low to moderate actin polymerization (red), no pseudopodia, and predominant nuclear $ER\alpha$ localization (green) with a lesser degree of cytoplasmic and membrane staining. (b and c) Cells treated with 10^{-10} M E_2 for 20 min. Cells display the formation of actin-containing dynamic membranes (red), including ruffles (B), pseudopodia (C) and a dramatic change in cell shape. The intense $ER\alpha$ (yellow) was observed in the membrane ruffles and in the peri-membrane region of the pseudopodia of E_2 treated cells. (d and e) ICI blocked the E_2 -induced morphology changes with little effects itself (Song *et al.* 2002c, reprinted from *Molecular Endocrinology* with permission of the publisher).

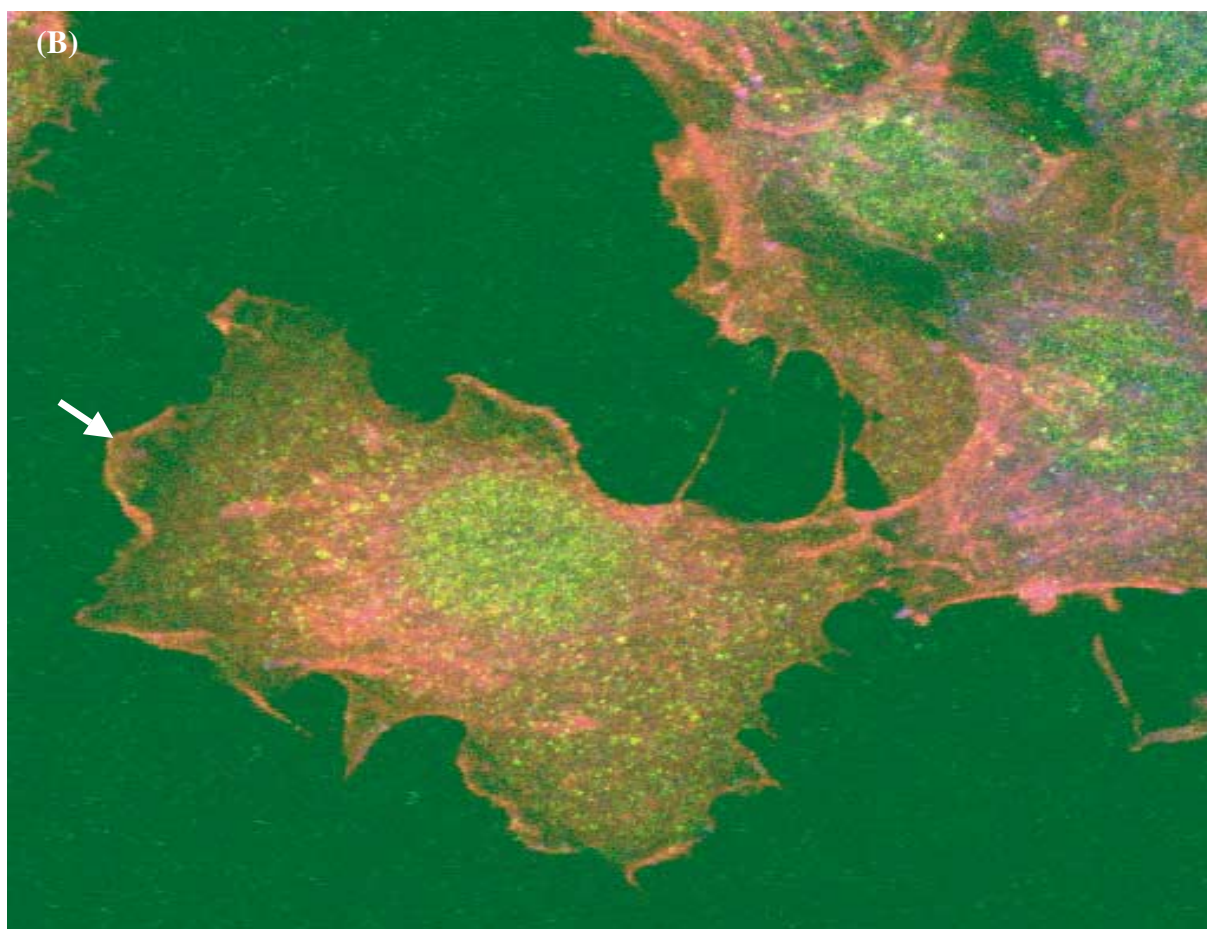


Figure 8 Continued

proliferation as evidenced by total cell counts (Song *et al.* 2002a). These data further support the function of the membrane ER to enhance cell proliferation.

Enhanced non-genomic effects in LTED cells

From the data reviewed, we conclude that membrane-related ER α plays a role in cell proliferation and in activation of MAP kinase. It appeared likely then that LTED cells might exhibit enhanced functionality of the membrane ER α system. As evidence of this, we examined the ability of E₂ to cause the phosphorylation of Shc in wild-type and MCF-7 cells and also to cause association of Shc with the membrane ER α . As shown in Fig. 9A and B we demonstrated a marked enhancement of both of these processes in LTED as opposed to wild-type cells. At the present time, it is not clear what is responsible for enhancement of the non-genomic ER α -mediated process. Up-regulation of the amount of ER α is likely to be one factor responsible. We have shown by a variety of methods that there is a 4- to 10-fold elevation of ER α in

LTED cells. Whether other processes are involved in addition is not currently clear.

Summary of mechanisms for adaptive hypersensitivity

Our current working model to explain adaptive hypersensitivity can be summarized as follows. LTED causes a 4- to 10-fold up-regulation of the amount of ER α present in cell extracts. Rapid, non-genomic effects of E₂ such as the phosphorylation of Shc and binding of Shc to ER α are also enhanced in these cells. Taken together, these observations suggest that adaptive hypersensitivity is associated with an increased utilization of non-genomic, plasma membrane-mediated pathways. This results in an increased level of activation of the MAP kinase as well as the PI-3 kinase pathways. All of these signals converge on downstream effectors which are directly involved in cell cycle functionality and which probably exert synergistic effects at that level. As a reflection of this synergy, E2F1, an integrator of cell cycle stimulatory and inhibitory events, is hypersensitive to the

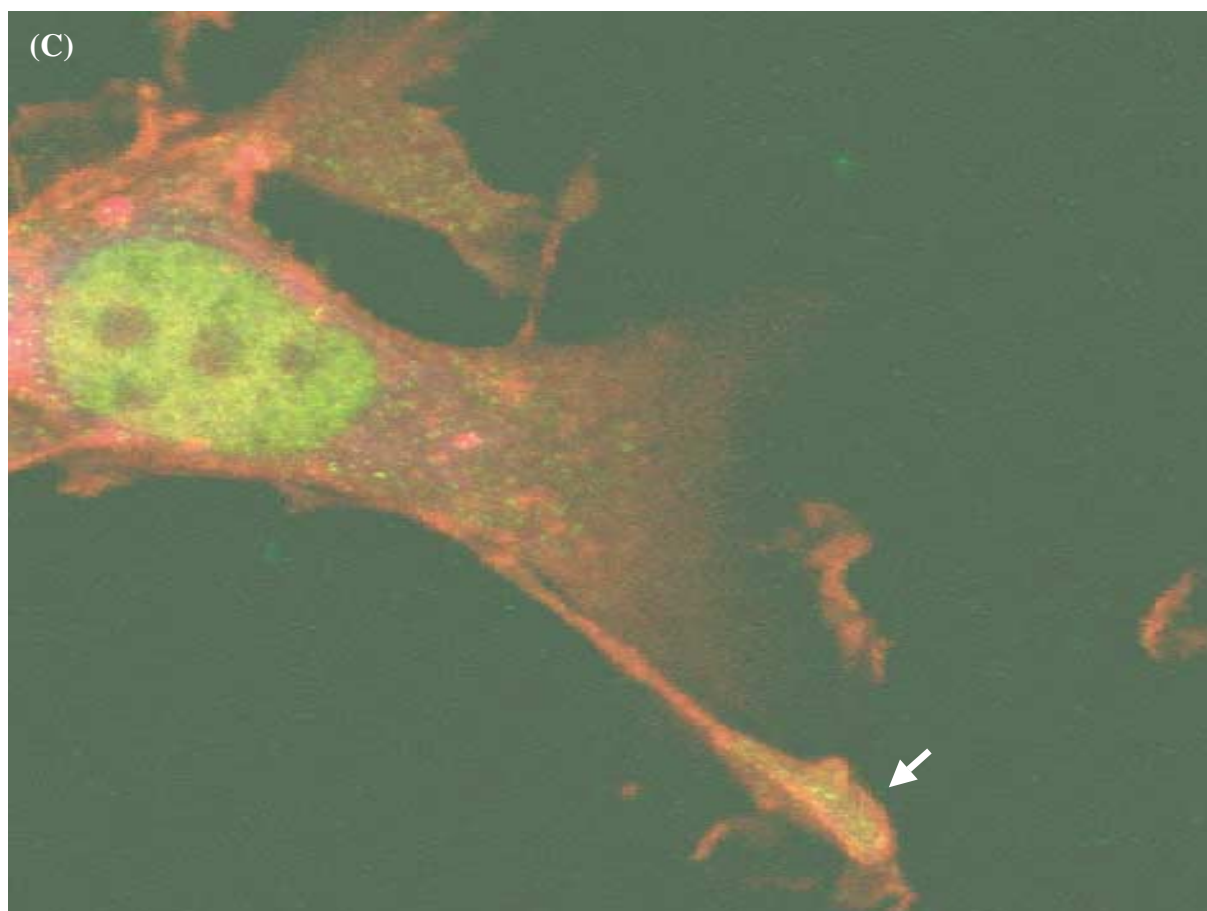


Figure 8 Continued

effects of E_2 in LTED cells (Yue *et al.* 2002). Thus, our working hypothesis at present is that hypersensitivity reflects upstream non-genomic $ER\alpha$ events as well as downstream synergistic interactions of several pathways converging at the level of the cell cycle. An increase in the basal level of transcription of $ER\alpha$ -regulated genes may also be involved in the process but does not represent the proximate cause of hypersensitivity since transcriptional events respond to E_2 with similar dose-response curves in wild-type and LTED cells.

Relevance of adaptive hypersensitivity

It is clear that primary endocrine therapies can exert pressure on breast cancer cells that causes them to adapt. Accordingly, these cells manifest inherent plasticity. We postulate that certain patients may become resistant to tamoxifen as a result of developing hypersensitivity to the estrogenic properties of tamoxifen (Santen 1996). This might explain the superiority of clinical responses in patients receiving aromatase inhibitors

as opposed to tamoxifen. If breast cancer cells are exceedingly sensitive to small amounts of E_2 or to the estrogenic properties of tamoxifen, one therefore needs highly potent aromatase inhibitors to block estrogen synthesis.

Development of third-generation aromatase inhibitors

A major focus over the past two decades has been on the development of highly potent aromatase inhibitors (Yue & Santen 1996, Santen & Harvey 1999). Second- and third-generation inhibitors have now been developed which are 100- to 1000-fold more potent than the first-generation inhibitor, aminoglutethimide, and specific to the aromatase enzyme. Isotopic kinetic techniques demonstrate that the newer inhibitors block all but 1–3% of aromatase activity and highly sensitive bioassays for E_2 demonstrate a 95% reduction of circulating E_2 levels (Klein *et al.* 1995, Geisler *et al.* 2002).

If the adaptive hypersensitivity hypothesis were correct, third-generation aromatase inhibitors would be more effective

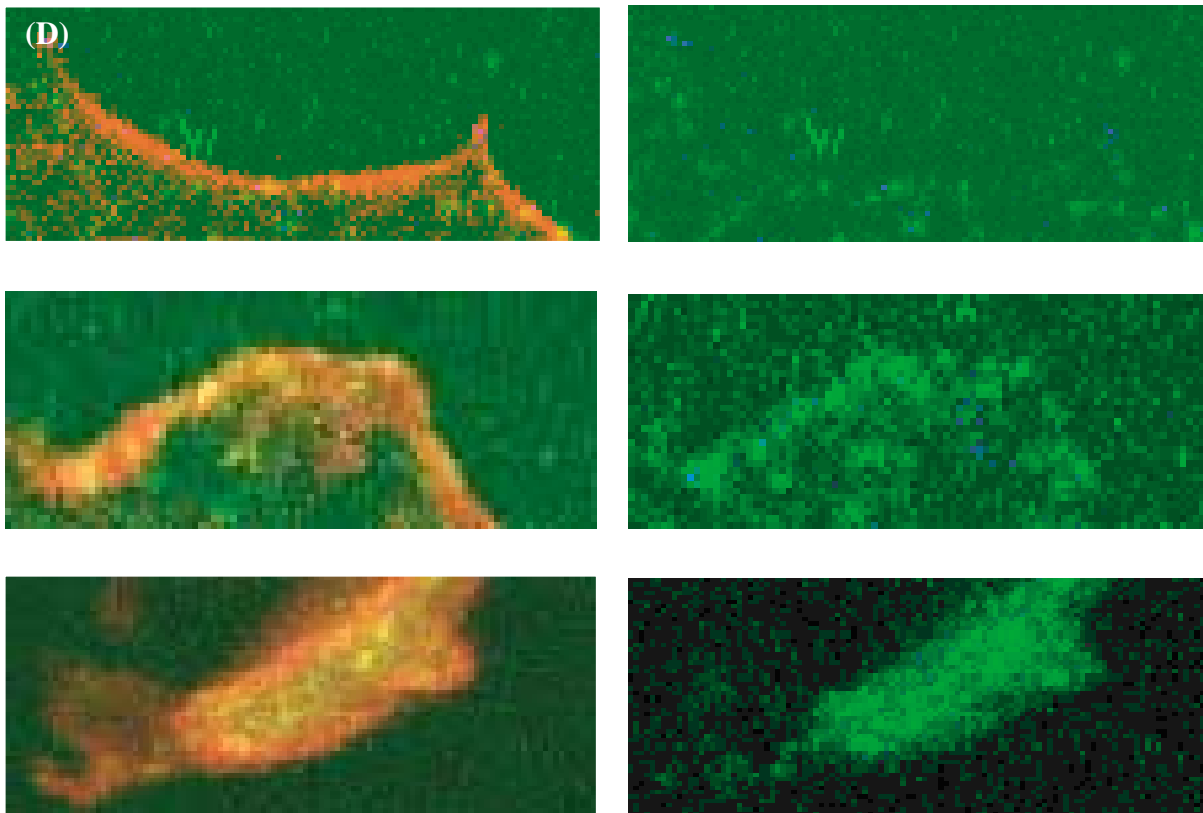


Figure 8 Continued

tive than first-generation agents for treatment of patients with breast cancer. One would also expect aromatase inhibitors to be superior to tamoxifen, since this agent exerts partial agonist activity which is enhanced by the adaptive hypersensitivity process. Both expectations have been substantiated in clinical trials. Five large randomized trials demonstrate the greater efficacy of third-generation aromatase inhibitors over tamoxifen for first-line therapy in advanced disease (Bonnetterre *et al.* 2000, Nabholz *et al.* 2000, Milla-Santos *et al.* 2001, Mouridsen *et al.* 2001, Paridaens *et al.* 2000, Santen 2002). Two large randomized trials demonstrate the superiority of aromatase inhibitors over tamoxifen in the adjuvant as well as neo-adjuvant settings (Dixon *et al.* 1999, 2000, 2001, Ellis *et al.* 2001, ATAC Trialists' Group 2002, Buzdar 2002).

Breast cancer prevention

The aromatase inhibitors might also be superior to the SERMs for breast cancer prevention. This contention is based upon our concepts regarding the precise mechanisms for estrogen-induced carcinogenesis. The molecular basis for

this process is not fully understood and represents an area of substantial debate. The most commonly held theory is that several mutations of key genes involved in cell proliferation or DNA repair must accumulate to result in invasive breast cancer (Preston-Martin *et al.* 1990, Feigelson *et al.* 1996) (Fig. 10). Most investigators agree that estrogens serve a promotional effect to cause proliferation of breast tissue and propagation of cells containing genetic mutations. The general hypothesis, as stated by Henderson & Feigelson (2000) holds that estrogens can also initiate mutations leading to breast cancer by increasing the rate of cell proliferation. As the rate of cell division increases, the chance for errors in DNA replication is augmented and the time available for DNA repair decreases.

An emerging theory suggests that additional events involving genotoxic E_2 metabolites participate in the carcinogenic process (Yager & Liehr 1996, Cavalieri *et al.* 2000, Yager 2000). These genotoxic products cause point mutations and serve as a means to initiate breast cancer. The enzyme cytochrome p450 1B1 metabolizes estrone and E_2 to their 4-hydroxylated, catechol-estrogen derivatives. These in turn are metabolized to the 3,4- E_2 (estrone)-quinones. These

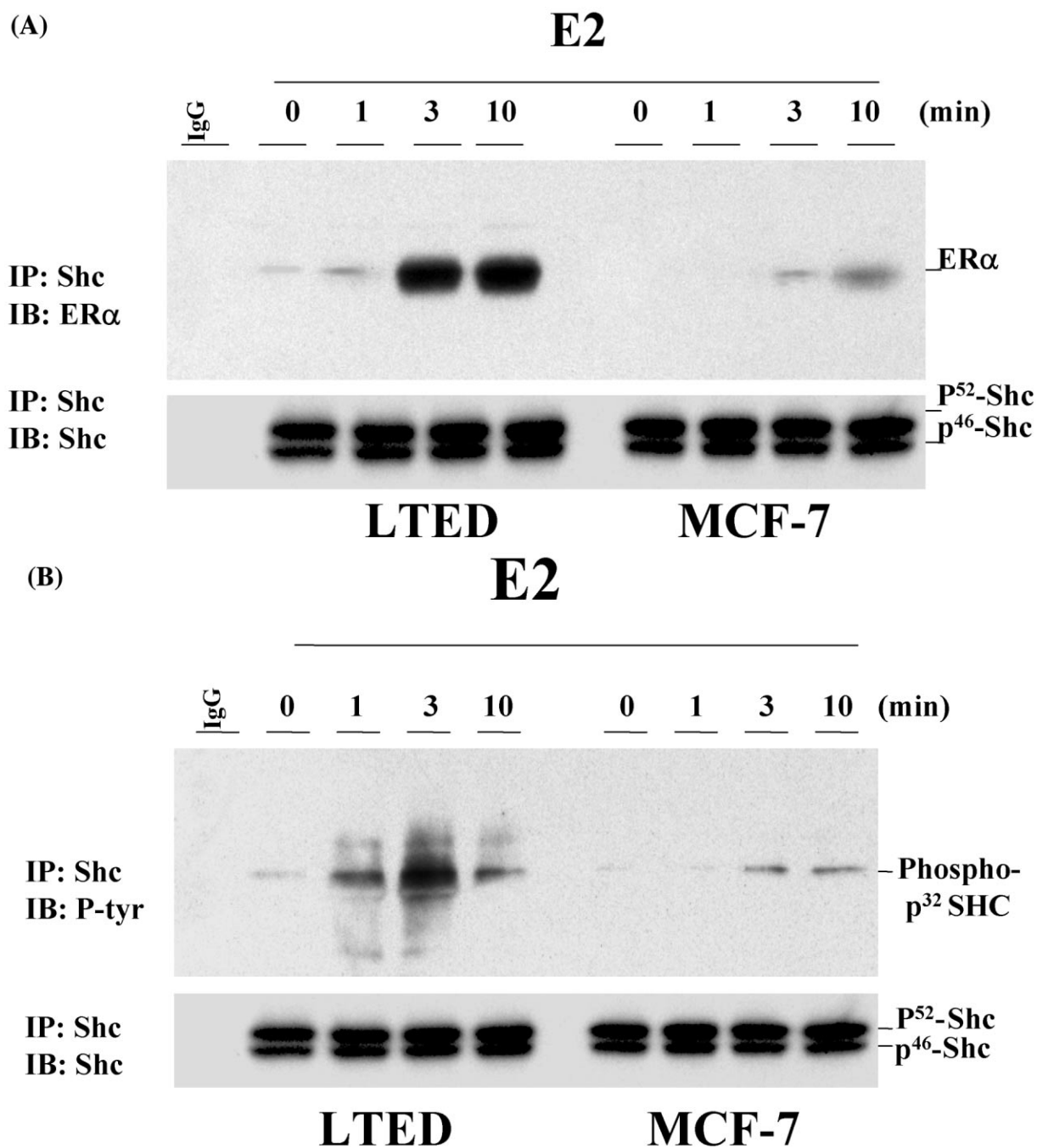


Figure 9 (B) E₂ rapidly induced the phosphorylation of Shc in LTED and MCF-7 cells. Cells were treated as in (A) above for the times indicated. Subsequently, an anti-Shc antibody was used to immunoprecipitate the complexes. Western blots were then performed using an anti-P-tyr antibody to demonstrate tyrosine phosphorylation. Protein loading is shown on the bottom panel. IP indicates immunoprecipitation, IB indicates immunoblot using western blot technique. Reprinted from *Molecular Endocrinology* with permission of the publishers.

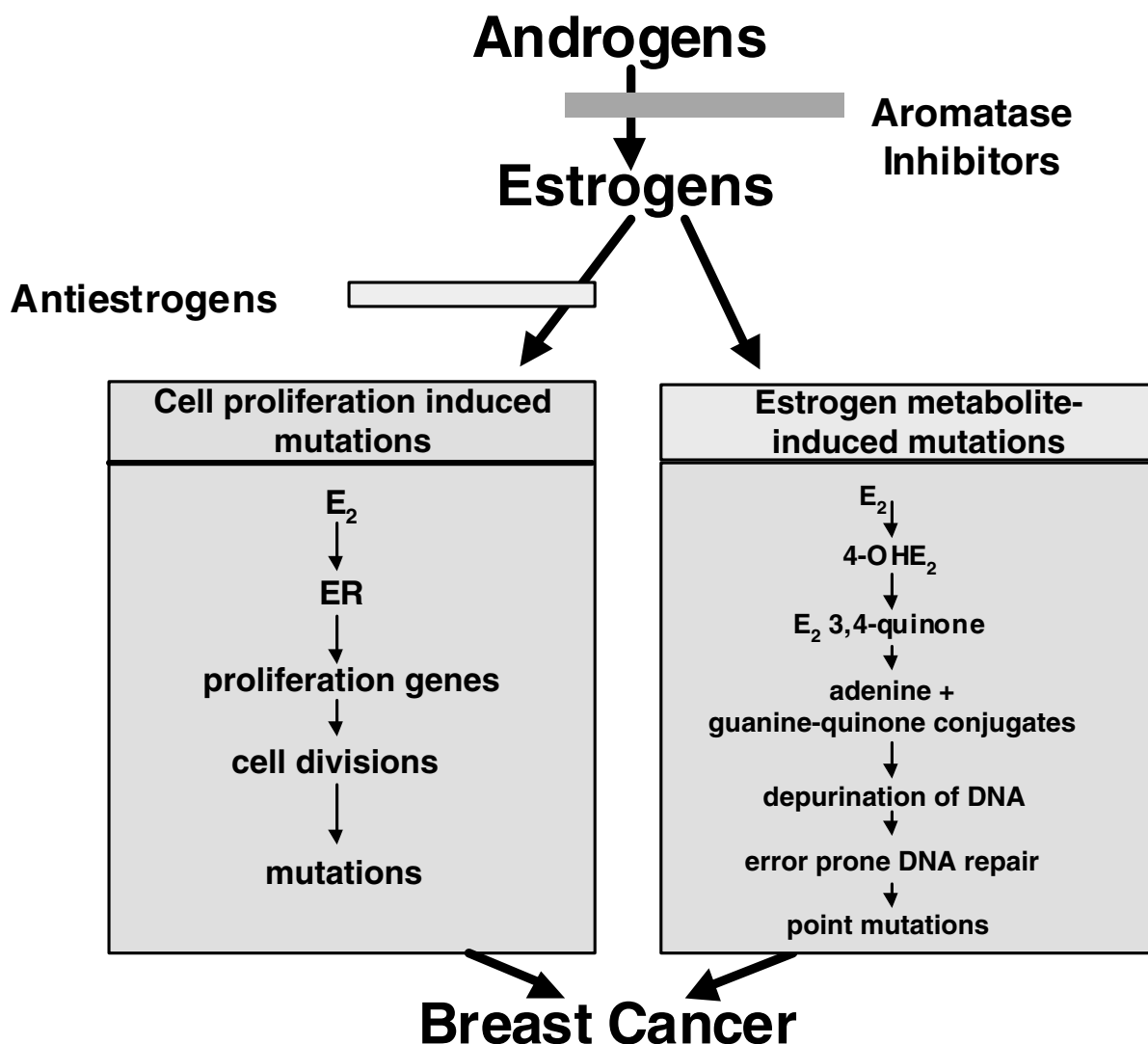


Figure 10 Diagrammatic representation of the two mechanisms whereby estrogens may cause breast cancer. The left side of the diagram demonstrates that E_2 may bind to the ER and stimulate transcription of genes involved in cellular proliferation. With a sufficient number of cell divisions, mutations occur which are ‘promoted’ by the effects of E_2 on cell proliferation. The right side of the diagram illustrates the metabolic pathway whereby E_2 is converted into genotoxic metabolites. E_2 is converted into 4-hydroxy-estradiol which can then be converted into the 3,4-quinone. This molecule is highly reactive and binds covalently to guanine or adenine on DNA and causes depurination. With error-prone DNA repair, mutations occur. With a sufficient number of critical mutations, breast cancer may occur. The antiestrogens block the pathways shown on the left side of the diagram and aromatase inhibitors block pathways on both sides (Santen 2002, reprinted from the *Journal of Clinical Endocrinology and Metabolism* with kind permission of the publishers).

highly reactive species bind to guanine or adenine molecules in the DNA molecule and result in depurination. Error-prone DNA repair or replication-mediated insertion of alternate nucleotides on the depurinated segment result in point mutations on critical genes (Chakravarti *et al.* 2000).

Support for the genotoxic hypothesis emanates from the direct demonstration in breast tissue that products of E_2 -quinone depurination are present in high concentration

(Rogan 2003). The direct demonstration that E_2 can induce mutations in V-79 cells also directly supports this hypothesis (Kong *et al.* 2000). The V-79 cell assay is similar to the Ames carcinogenesis assay and is designed to detect the mutagenesis of various agents. E_2 can also induce mutations, loss of heterozygosity in key regions of DNA and anchorage-independent growth (a sign of cell transformation) in the benign MCF-10 cell line (Russo *et al.* 2001). Proponents of

the quinone-depurination hypothesis suggest that this mechanism is responsible for initiation of mutations whereas the proliferative effects of E_2 mediate tumor promotion. Based upon current evidence, we postulate that the cell proliferation and genotoxic pathways work in an additive or synergistic fashion to cause breast cancer.

Evidence is currently available from patients that blockade of the proliferative pathway reduces the incidence of breast cancer (Duffy & Jackson 2001, ATAC Trialists' Group. 2002). Two SERMs, tamoxifen and raloxifene, reduce the incidence of breast cancer in women at high risk of this disease (Fisher *et al.* 1998, Cummings *et al.* 1999). These agents act by binding to the ER and blocking cellular proliferation. This would abort the promotional and perhaps also the initiational effects of E_2 . If the genotoxic metabolism hypothesis were correct, use of aromatase inhibitors to block E_2 production should be more effective to prevent breast cancer than the SERMs. A large recent clinical trial (the ATAC trial) also provides support for this possibility (ATAC Trialists' Group 2002, Buzdar 2002).

Findings in the ATAC trial require interpretation based upon past observations with tamoxifen. The first clinical studies to demonstrate the efficacy of tamoxifen for the prevention of breast cancer were in the adjuvant therapy setting (Fisher & Redmond 1991). Women with the initial diagnosis of breast cancer underwent lumpectomy and local irradiation. In order to abrogate the effects of occult distal metastases, they were also given tamoxifen for a period of 5 years. This therapy not only reduced the number of recurrences of the primary tumor but also the incidence of new tumors in the contralateral breast. These observations provided the first demonstration that the SERMs prevent breast cancer. Based upon these observations, large trials were initiated in women without cancer, which conclusively demonstrated that tamoxifen or raloxifene reduce the incidence of breast cancer by 50–75% (Fisher *et al.* 1998, Cummings *et al.* 1999).

Data from an adjuvant clinical trial comparing tamoxifen with the aromatase inhibitor anastrozole, now allow assessment of their relative efficacy for the prevention of contralateral breast cancer. This study, called the ATAC trial, compares the use of tamoxifen alone, anastrozole alone, and the two in combination. ATAC is an acronym for anastrozole and tamoxifen, alone and in combination. A group of 8366 women with ER-positive breast cancer were randomized to receive 5 years of therapy with 20 mg tamoxifen, 1 mg anastrozole or the combination of the two drugs daily. Data from the first 4 years of follow-up have been published (Buzdar 2002). A striking and statistically significant ($P < 0.05$) reduction in the number of contralateral invasive breast cancers in the anastrozole-alone arm were observed. Forty contralateral invasive and *in-situ* tumors were detected in the tamoxifen-alone arm and 25 in the anastrozole-alone arm. Of interest was the observation that tamoxifen appeared to counteract the ameliorative effect of anastrozole in the combined treatment arm.

The observations in the ATAC trial support (but do not prove) the hypothesis that the cell proliferative and genotoxic pathways act in concert to cause breast cancer (Buzdar 2000). Tamoxifen (and raloxifene) blocks only the cell proliferative pathway whereas the aromatase inhibitor inhibits both. On this basis, one would expect that women in the aromatase inhibitor arm would continue to experience a lower incidence of breast cancer over time. This will be verified as the ATAC trial matures. In addition, one might expect that the aromatase inhibitor would prevent ER-positive as well as ER-negative breast cancers. The SERMs are known to prevent ER-positive tumors exclusively. It is important to understand that the genotoxic effects of E_2 can explain tumor development without postulating the need for an ER. No data are as yet available about the receptor status of the contralateral tumors which have occurred in the ATAC trial.

A puzzling feature of the ATAC trial is that tamoxifen appeared to blunt the favorable effects of anastrozole, both for contralateral tumor formation and for diminution of rate of recurrence of the primary tumor (ATAC Trialists' Group 2002). Our hypothesis regarding adaptive hypersensitivity would serve as a potential explanation for this observation. We propose that exposure to tamoxifen induces hypersensitivity to its intrinsic estrogenic effects and to estrogen itself. Experiments by Jordan and colleagues (Gottardis *et al.* 1989) and Osborne & Fuqua (1994) in nude mice clearly demonstrated that long-term exposure to tamoxifen results in tumor stimulation via its estrogenic effects. As evidence that tamoxifen had become an estrogen, tumor regrowth under these circumstances could be blocked by the pure antiestrogen, fulvestrant. In the ATAC trial, the dual effects of E_2 deprivation with anastrozole and blockade of estrogen action with tamoxifen would exert pressure for tumors to adapt. As a consequence, these tumors would become hypersensitive to the estrogenic properties of tamoxifen. If correct, this hypothesis would explain the blunting of the ameliorative effect of anastrozole on tumor recurrence. With respect to prevention, tamoxifen would stimulate proliferation of breast cells and serve as a surrogate for E_2 on the proliferative pathway. This effect would serve to dampen the protective effects of tamoxifen.

Model systems to study genotoxic pathways

The concept of the genotoxic pathway, while controversial, is quite important for the design of breast cancer prevention strategies. Our laboratory is focusing on model systems to demonstrate that this pathway is indeed operative in the genesis of breast cancer (Yue *et al.* 1998). Aromatase-transfected human breast cancer cells can convert testosterone to guanine- E_2 (estrone) depurinated products and this effect is abrogated with the aromatase inhibitor letrozole. We utilize the ER α knock out, Wnt-1 double transgenic mouse model to

examine the genotoxic effects of E₂ in the absence of a functional ER (Bocchinfuso *et al.* 1999, Yue 2003a). Preliminary data from this model demonstrate the presence of genotoxic metabolite formation (Badawi *et al.* 2001). More importantly, castration at day 15 reduced the incidence of breast tumors in animals lacking a functional ER. Further studies with this model are expected to demonstrate that E₂ add-back in castrate animals will enhance the rate of tumor formation. Studies by Russo *et al.* (2001) and Kong *et al.* (2000) have directly demonstrated the genotoxicity of E₂ *in vitro* in breast cancer cells and its ability to induce neoplastic transformation of benign MCF-10 breast cancer cells. Taken together, these studies provide a mechanistic basis for the superiority of aromatase inhibitors over the SERMs for prevention of breast cancer.

Summary

Our data and that of others support the concept that breast cancer cells are plastic and adapt to the pressures induced by various endocrine therapies. This results in development of hypersensitivity to E₂ and to the estrogenic properties of tamoxifen. These mechanistic events provide a cogent explanation for the superiority of aromatase inhibitors over the SERMs for treatment of breast cancer. Adaptive hypersensitivity and the genotoxic effects of E₂ may also provide a mechanistic basis for the superiority of the aromatase inhibitors in the prevention of breast cancer. These concepts, when translated into strategies to use highly potent aromatase inhibitors for breast cancer prevention, might ultimately allow a substantial reduction in breast cancer incidence. For this reason, we believe that study of the genotoxic effects of E₂ represent a highly important focus for future studies regarding the etiology of breast cancer.

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