Inhibition of Insulin-like Growth Factor Signaling Pathways in Mammary Gland by Pure Antiestrogen ICI 182,780[1]

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ABSTRACT

The antiestrogens ICI 182,780 (ICI) and tamoxifen are clinically useful in the treatment of estrogen receptor-positive breast tumors. We assessed the *in vivo* effects of ICI, tamoxifen, and estradiol on the insulin-like growth factor (IGF) signaling pathway in the rat mammary gland. ICI significantly decreased the size of the lobular structures, Ki-67 labeling index, and *insulin-like growth factor binding protein* (*IGFBP*)*-2* and *IGFBP-5* gene expression. Treatment of rats with 1, 1.5, and 2 mg of ICI/kg body weight/week resulted in a 2-, 7-, and 8-fold increase in IGFBP-3 transcripts. High doses of ICI increased mammary *IGF-1* gene expression by 2-fold ($P < 0.01$) but decreased IGF-1R and its autophosphorylation to ~30% of the control mammary gland. IRS-1, IRS-2, and c-Raf-1 levels in the ICI-treated mammary glands were approximately 30, 15, and 40% of controls, respectively. Basal phosphorylation of IRS-1, Akt-1, and the p85 subunit of phosphatidylinositol 3-kinase (PI-3K) were low but detectable after ICI treatment. Despite a significant reduction in levels of IGF-1R, IRS-1, and IRS-2 phosphorylation, phospho p42/p44 MAPK levels were only slightly decreased. Tamoxifen-induced growth inhibition was associated with slight stimulation of *IGFBP-3* gene expression and reduction in IRS-2 levels. Basal phosphorylation of IGF-1R, IRS-1, and p85 subunit of PI-3K was decreased by tamoxifen. Estradiol-induced epithelial cell proliferation was associated with inhibition of *IGFBP-3* gene expression, stimulation of *IGFBP-2* gene expression, and increases in IGF-1R, IRS-1, IRS-2, and c-Raf-1 levels. Although basal phosphorylation of IGF-1R, IRS-1, IRS-2, Akt-1, and the p85 subunit of PI-3K was significantly increased by estradiol, basal phospho p44/42 MAPK was significantly reduced. The data indicate that in addition to their classic actions, antiestrogens have major effects on IGF signaling pathways.

INTRODUCTION

Epidemiological studies have shown a link between IGF-1[3] and risk of breast cancer. Among premenopausal women <50 years of age, there was a 4.5-fold relative risk of breast cancer in the highest quartile of plasma IGF-1 compared with the lowest quartile (1). IGFs are mitogenic and antiapoptotic agents for breast epithelial cells *in vitro* (2, 3). IGFs exert their effects through IGF-1R. Target disruption of the IGF-1R by either antibody against IGF-1R or antisense to the IGF-1R restricts breast cancer cell proliferation both *in vivo* and *in vitro* (4). The IGF-1R is found in a high percentage of primary human mammary tumors, and this expression is positively correlated with the ER level (5). *In vivo* studies have demonstrated that the development of terminal end bud of mammary gland was impaired in the absence of IGF-1 (6–8). IGF-1 acts with estrogens to stimulate ductal morphogenesis (6). Treatment of mice with estradiol had no effect on mammary development in IGF-1 null mice. The effect of estrogen on cell growth may be mediated by the up-regulation of IGF-1R expression (9, 10), IRS-1 and IRS-2 (11), or by an increase in IGF-1-induced IGF-1R, IRS-1, and ERK1/ERK2 phosphorylation (12). Furthermore, estrogens induced redistribution of IGF-1R to the cell surface (12).

IGF action is modulated by IGFBPs, which are potential mediators of apoptosis (13). Six high-affinity IGFBPs have been described (13–15). There is clear evidence that they modulate activity of IGFs. The effect of estrogens on breast cell growth may be mediated by down-regulation of the inhibitory IGFBP-3 (16). IGFBP-3 has been shown to be related inversely to risk, whereas IGF-1 was positively related to risk of breast cancer (1).

IGF binding induces IGF-1R autophosphorylation. Phosphorylated IGF-1R phosphorylates IRS-1, IRS-2, and Shc and activates the signal transduction pathways, such as PI-3K and Ras/Raf/MAPK (17). The first signaling cascade involves activation of PI-3K and subsequent formation of phosphatidylinositol 3-phosphate, which can serve as a signal for cell growth. The Ras/Raf/MAPK pathway involves the ERK1 and ERK2 (MAPKs). Phosphorylation of IRS-1 by the IGF-1R results in the formation of an IRS-1-Grb2-Sos complex, which activates Ras. Activated Ras p21 binds Raf-1 and activates Raf-1, which results in the phosphorylation and activation of ERKs, which in turn transmit a signal to the nucleus (18). Ras/Raf/MAPK and

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[3] The abbreviations used are: IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; ER, estrogen receptor; ERK, extracellular signal-related kinase; IGFBP, IGF binding protein; PI-3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ICI, ICI 182,780; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PI-3K have been identified as playing important roles in IGF-1R-induced cellular proliferation and the inhibition of apoptosis. The Ras/Raf/MAPK pathway was thought to primarily mediate the cell proliferative response to growth factors such as IGFs, whereas the PI-3K pathway which activates Akt was primarily implicated in mediating antiapoptotic effects of IGFs (19).

A high proportion of primary breast cancers contain the ER and require estrogenic activity for tumor growth. Current therapies have been directed toward interruption of estrogen by oophorectomy or the use of antiestrogens (20, 21). The antiestrogen drug tamoxifen has proven to be clinically useful for the treatment of metastatic ER-positive tumors (22, 23). It has been proposed that the inhibitory effect of antiestrogens on IGF-1 expression contributes to their antiproliferative activity (24–26). Unfortunately, the overwhelming majority of tumors progress to a phenotype characterized by resistance to tamoxifen, thus restricting clinical utility of this drug (20). The pure antiestrogen ICI (23) is effective in some patients after disease progression on tamoxifen (21). ICI has been shown recently to act as a growth inhibitor, even in the complete absence of estrogen stimuli (16, 27), and to actively regulate gene expression in a direction opposite to that of estrogens (16). We and others have shown that ICI had a stronger antineoplastic activity than tamoxifen and also lacks the uterine side effects of tamoxifen (28).

Here we report influences of antiestrogens on IGF-1 signal transduction. Because the IGF system plays an important role in breast cancer cell proliferation, metastasis, and apoptosis, the described activities of tamoxifen and ICI may contribute to their antiproliferative and antineoplastic activity seen clinically and in animal models.

MATERIALS AND METHODS

Animal experiments were approved by the Local Animal Care Committee. Ovary-intact female Sprague Dawley rats, 60 days of age at the beginning of the experiments, were obtained from Charles River (Boston, MA). To investigate the effects of ICI on expression of the IGF system in the mammary gland, rats were weekly injected with 1 mg ($n = 15$), 1.5 mg ($n = 15$), and 2 mg ($n = 15$) ICI (Astra-Zeneca Pharmaceuticals, London, United Kingdom) per kg body weight in castor oil for 3 weeks. Control rats ($n = 15$) received an injection of an equal volume of castor oil. For estradiol studies, groups of rats ($n = 15$) were implanted with 0.5-cm silastic tubes (0.04-inch inside diameter; Dow Corning, Midland, MI) containing 17β-estradiol (Sigma Chemical Co., St. Louis, MO) on the back of their necks. Control rats ($n = 15$) experienced the same surgical implantation with empty silastic tubes. On the basis of work published previously (29), the rate of release of 17β-estradiol from silastic implants was documented to be 2.4 μg/cm/day. To examine the effects of tamoxifen on expression of genes in the IGF system, rats ($n = 15$) were implanted with either empty silastic tubes (0.12-inch inside diameter; Dow Corning) or 4-cm silastic tubes containing tamoxifen (Sigma Chemical Co.) on the back of their necks. The rate of release was ~25 μg/cm/day. Animals were sacrificed by carbon dioxide at the end of the experiment. The mammary tissue was excised, trimmed, and frozen in liquid nitrogen and stored at −70°C for RNA extraction. Part of the mammary tissue was fixed in 10% buffered formalin for histochemical studies.

Immunohistochemistry and Histology. Fixed mammary tissue was routinely processed in a tissue processor and embedded in paraffin. Sections of 5 μm were cut and stained with H&E. Examination of the slides was performed by light microscopy. The ImmunoCruz Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for immunohistochemical study. Briefly, the slides were deparaffinized, rehydrated in water, and incubated with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. To examine expression of Ki-67, antigens were retrieved by heating the slides in citrate buffer (pH 6) for 5 min. After preincubation with normal serum for 20 min at room temperature, the primary antibody was applied (2 μg/ml) and incubated overnight at 4°C. The section was then incubated with the appropriate biotinylated secondary antibody at 1:500 dilution, followed by peroxidase-conjugated streptavidin complex according to the manufacturer's instructions, and 3,3′-diaminobenzidine. The section then was counterstained with hematoxylin. Between each change of incubation, the sections were rinsed three times in PBS for 5 min each. To evaluate the Ki-67 labeling index, 500 epithelial cells were counted for each group in randomly chosen fields at ×400. The Ki-67 labeling index was expressed as the number of clearly labeled Ki-67-reactive nuclei in 500 cells counted. The significant difference was determined by Student's *t* test.

Western Blotting. To determine the changes in the levels of the p85 subunit of PI-3K, c-Raf-1, IGF-1R, IRS-1, IRS-2, phospho Akt-1, and phospho p44/42 MAPK, mammary tissue was homogenized in lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, and 100 μM $NaVO_4$). Proteins were subjected to Western blot analysis as described (30). Blots were incubated with indicated primary antibodies and horseradish peroxidase-conjugated donkey antimouse or antirabbit secondary antibody (1:7500). Blots were visualized with a chemiluminescent detection system as described by the manufacturer (ECL; Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). Rabbit anti-PI-3K p85, rabbit anti-c-Raf-1, mouse anti-α-tubulin, and rabbit anti-IGF-1R β antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-IRS-1, rabbit anti-IRS-2, and mouse anti-phosphotyrosine 4G10 (Thr-202 and Tyr-204) antibodies were obtained from Upstate Biotechnology (Lake, Placid, NY). Mouse anti-phospho-specific MAPK and rabbit anti-phospho-Akt-1 (Ser-473) antibodies were from New England BioLabs (Beverly, MA). Anti-α-tubulin antibodies were used at a final concentration of 0.5 μg/ml. Other antibodies were diluted into Tris-buffered saline Tween 20 (TBST) solution at a final concentration of 1 μg/ml, as recommended by the manufacturers.

Autophosphorylation of IGF-1R and tyrosyl phosphorylation of IRS-1 and IRS-2 were determined by immunoprecipitation of total cellular lysates using anti-IGF-1R β, IRS-1 and IRS-2, respectively. Briefly, 500 μg of total cellular proteins were incubated with 2 μg of primary antibody for 1 h at 4°C. Immunoprecipitates were collected using protein A/G Plus-Agarose (Santa Cruz Biotechnology). After washing four times with lysate buffer, the pellets were resuspended in electrophoresis sample buffer and boiled for 3 min. Immunoprecipitated proteins were analyzed by Western blotting using

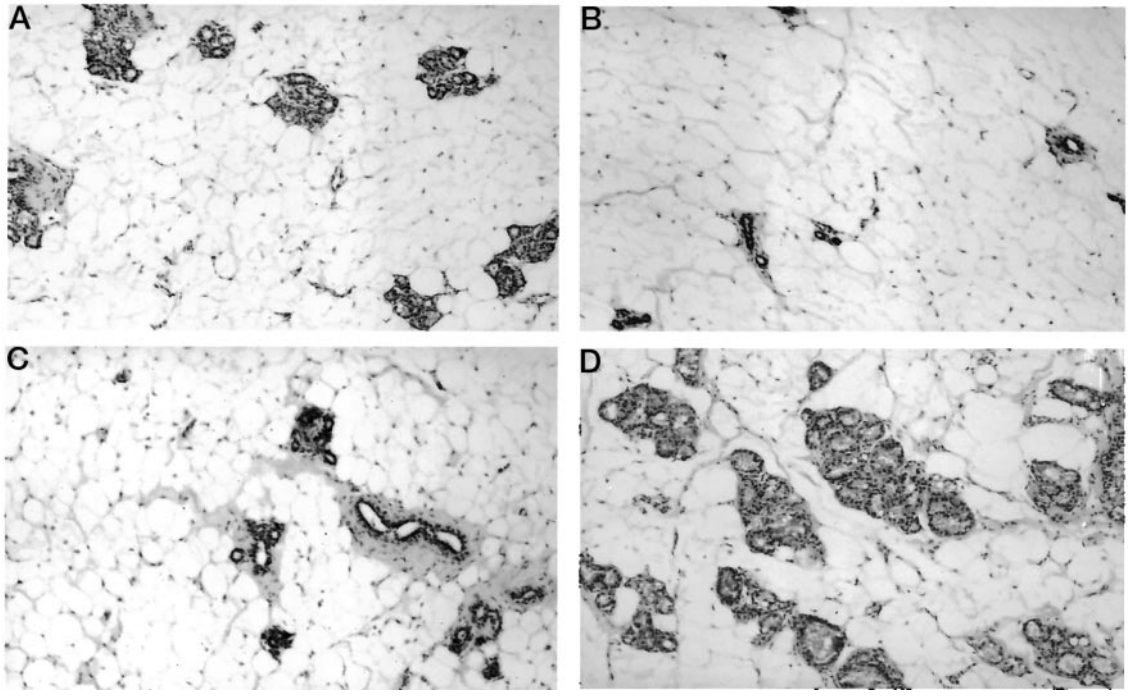

Fig. 1 Mammary gland histology in rats treated with vehicle (*A*), ICI (2 mg/kg body weight/week; *B*), tamoxifen (100 μg/day; *C*), or estradiol (1.2 μg/day; *D*). ICI and tamoxifen caused marked atrophy of the mammary gland, small atrophic alveoli, lined by atrophic and low cuboidal cells. Estradiol stimulated lobuloalveolar and ductal growth, as well as the secretory activity of acinar cells. H&E stain. ×400.

mouse anti-phosphotyrosine antibody (clone 4G10; Upstate Biotechnology).

Northern Blotting. Total RNA was isolated from mammary tissue, and Northern blotting was performed as described (25). Blots were hybridized with IGF-1 (31) and IGFBP (1–5, 14) cDNAs. To control for equal RNA loading, blots were rehybridized with GAPDH cDNA (American Type Culture Collection, Manassas, VA). Quantitative analysis of gene expression was accomplished by scanning autoradiograms and densitometry. For each lane, the sum of the density of bands corresponding to transcripts hybridizing with the probe under study was calculated, and we normalized the amount of RNA loaded.

Statistical Analysis. Differences in the above-mentioned parameters were analyzed by either Mann-Whitney *U* test or Student's *t* test.

RESULTS

Control mammary glands had a sparse cluster of epithelial tubules surrounded by a small amount of connective tissue, which was in turn embedded in a large fat pad. The epithelial ducts had a small lumen, lined by cuboidal cells with dark-stained nuclei (Fig. 1*A*). Treatment of ovary intact animals with ICI resulted in a marked atrophy of the mammary glands. The ICI-induced pattern was characterized by decreased size of the lobular structures, which consisted of small atrophic alveoli, lined by atrophic and low cuboidal cells (Fig. 1*B*). Higher magnification shows that the acinar epithelial cells were apparently inactive, with diminished quantity of cytoplasm (data not shown). Similar histological observations were also seen when rats were treated with tamoxifen (Fig. 1*C*). Estradiol stimulated extensive lobuloalveolar and ductal growth, as well as the epithelial cells (Fig. 1*D*).

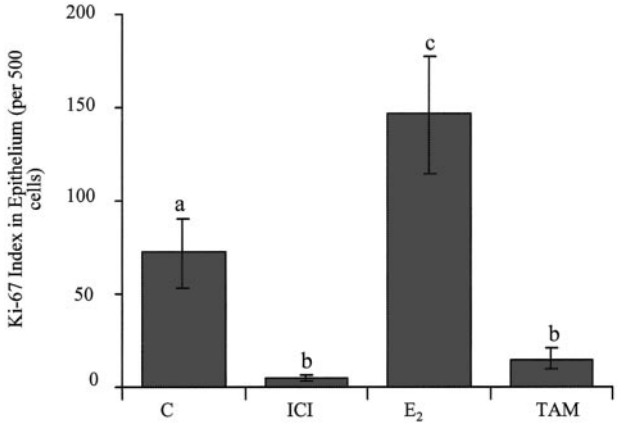

Fig. 2 Effects of treatment with vehicles (*C*), ICI (2 mg/kg body weight/week), tamoxifen (*TAM*; 100 μg/day), or estradiol (E_2; 1.2 μg/day) on Ki-67 labeling index of mammary epithelial cells. ICI and tamoxifen were effective in blocking Ki-67 labeling index of epithelial cells whereas estradiol stimulated it. *Columns* with different *letters* are significantly different from one another ($P < 0.01$). Data are expressed as the means; *bars*, SE.

We determined the effect of antiestrogens and estradiol on Ki-67 expression in mammary tissue. Fig. 2 shows the results of an experiment where mammary tissues were collected from rats

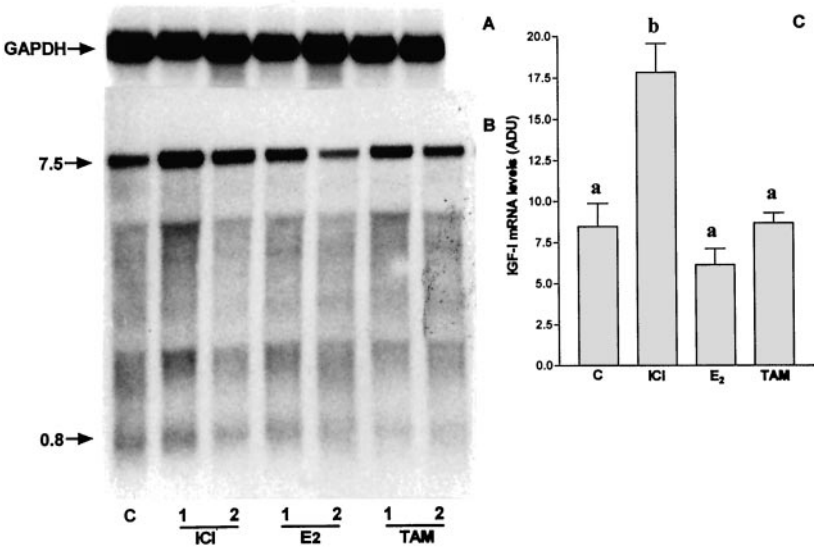

Fig. 3 Effects of ICI, tamoxifen (*TAM*), and estradiol (E_2) on mammary IGF-1 mRNA levels. Rats were treated with the indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day), and ICI (2 mg/kg body weight/week). Total RNA derived from mammary gland was subjected to Northern blotting. Blots were hybridized with GAPDH (*A*) and rat IGF-1 (*B*) cDNAs. Densitometric scanning of the IGF-1 bands are shown in *C*. *Columns* with different letters are significantly different from one another ($P < 0.01$); *bars*, SE. ICI significantly increased *IGF-1* gene expression whereas estradiol slightly inhibited it.

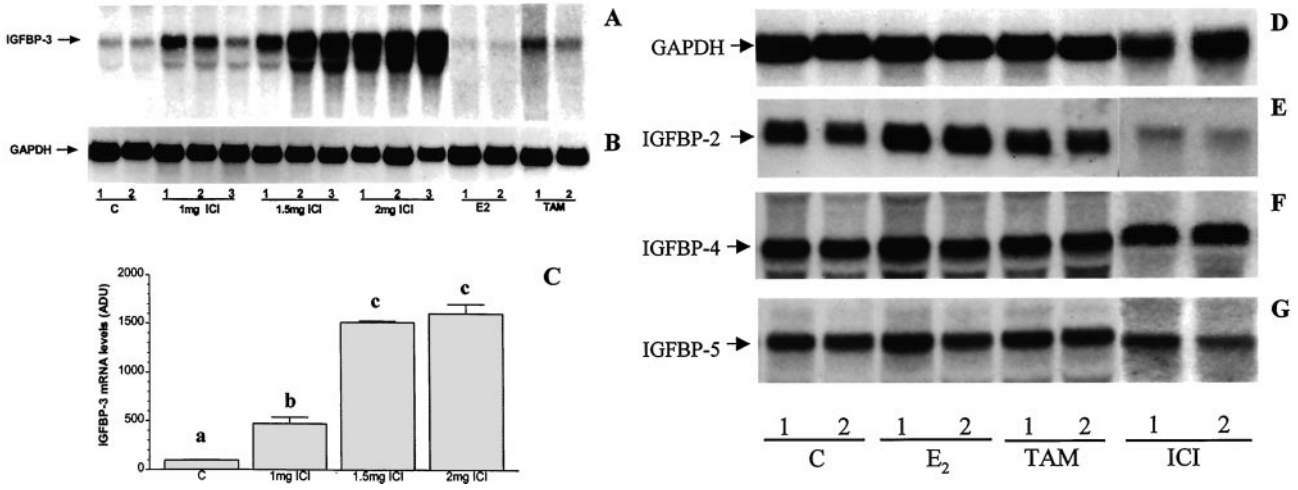

Fig. 4 Effects of ICI on mammary IGFBP-3, IGFBP-2, IGFBP-4, and IGFBP-5 mRNA levels. Rats were treated with the indicated concentrations of ICI, estradiol (*E2*; 1.2 μg/day), tamoxifen (*TAM*; 100 μg/day), and ICI (2 mg/kg body weight/week). Total RNA derived from mammary gland was subjected to Northern blot analysis. Blots were hybridized with rat IGFBP-3 (*A*), IGFBP-2 (*E*), IGFBP-4 (*F*), IGFBP-5 (*G*), and GAPDH (*B* and *D*) cDNAs. Densitometric scanning of the IGFBP-3 band is shown in *C*. *Columns* with different *letters* are significantly different from one another ($P < 0.01$); *bars*, SE. ICI induced *IGFBP-3* gene expression in a dose-dependent fashion. ICI significantly inhibited *IGFBP-2* and *IGFBP-5* gene expression ($P < 0.01$).

treated with vehicle, ICI, 17β-estradiol, and tamoxifen for immunohistochemical analysis of Ki-67 expression. Estradiol significantly increased ($P < 0.01$) whereas ICI and tamoxifen significantly decreased ($P < 0.01$) the number of epithelial cells expressing Ki-67.

Because IGF-1 is potent mitogen for normal and breast cancer cells, IGFBPs modulate activity of IGF-1 (32) and estradiol sensitizes ER-positive MCF-7 cells to the mitogenic effect of IGF-1 (4, 12), the effects of ICI on mammary *IGF-1* and *IGFBP* gene expression were examined. As shown in Fig. 3, ICI increased IGF-1 mRNA by 2-fold ($P < 0.05$), whereas estradiol and tamoxifen had no significant effect on *IGF-1* gene expression. ICI also induced *IGFBP-3* mRNA accumulation in a dose-dependent manner (Fig. 4*A*), whereas *IGFBP-2* and *IGFBP-5* gene expression were inhibited significantly ($P < 0.01$; Fig. 4, *E* and *G*). Estradiol significantly inhibited *IGFBP-3* ($P < 0.01$; Fig. 4*A*) and stimulated *IGFBP-2* gene expression (Fig. 4*E*). Tamoxifen mildly enhanced *IGFBP-3* gene expression (Fig. 4*A*).

Because overexpression of IGF-1R has been reported in variety of tumors (33) and *in vivo* overexpression of this receptor protected cells from apoptosis (34, 35), the effects of estradiol and antiestrogens on IGF-1R and its autophosphorylation were examined. ICI, but not tamoxifen, reduced IGF-1R levels

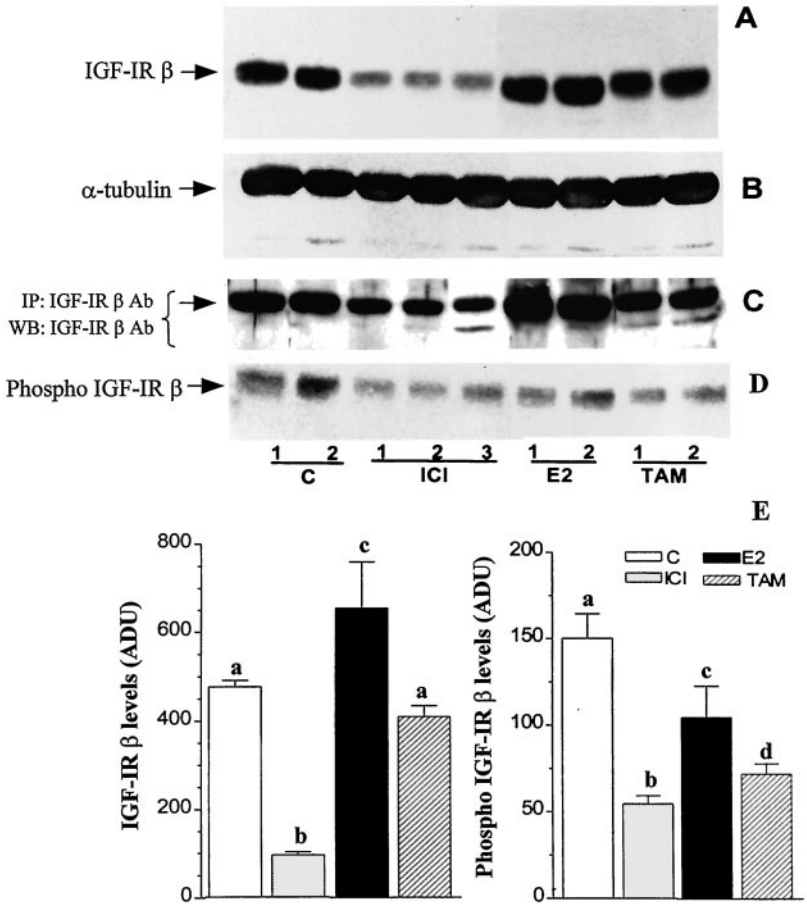

Fig. 5 Effects of ICI, tamoxifen (*TAM*), and estradiol (E_2) on mammary gland IGF-1 receptor and its autophosphorylation form. Rats were treated with the indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day), and ICI (2 mg/kg body weight/week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-IGF-1R β (*A*) and α-tubulin (*B*) antibodies. To determine the levels of IGF-1R autophosphorylation, tissue lysates were immunoprecipitated with anti-IGF-1R β antibody. After SDS-PAGE, the levels of IGF-1R β immunoprecipitated and autophosphorylated IGF-1R β subunit were detected by immunoblotting using IGF-1R β antibody (*C*) and antiphosphotyrosine antibody 4G10 (*D*), respectively. Densitometric scanning of the IGF-1R β subunit and autophosphorylated IGF-1R β subunit bands is shown in (*E*). *Columns* with different *letters* are significantly different from one another ($P < 0.01$); *bars*, SE. ICI significantly decreased both IGF-1R β and basal autophosphorylation of IGF-1R β whereas estradiol increased IGF-1R β levels.

compared with controls ($P < 0.01$; Fig. 5*A*). Estradiol significantly increased IGF-1R levels ($P < 0.01$). As shown in Fig. 5*D*, autophosphorylation of IGF-1R was significantly lower in ICI- and tamoxifen-treated mammary glands ($P < 0.05$). Despite high levels of IGF-1R protein, autophosphorylation of IGF-1R in estradiol-treated mammary tissue was slightly decreased (Fig. 5*D*).

To investigate whether antiestrogens and estradiol affected IRS-1 and IRS-2 levels and their tyrosyl phosphorylation, Western blot analysis was performed. As shown in Fig. 6, ICI significantly reduced both IRS-2 and IRS-1 levels ($P < 0.01$). Tamoxifen selectively reduced IRS-2 but not IRS-1 (Fig. 6, *A* and *B*). Basal tyrosyl phosphorylation of IRS-1 was significantly reduced after ICI and tamoxifen treatments ($P < 0.01$; Fig. 6*F*). Basal tyrosyl phosphorylation of IRS-2 in ICI- and tamoxifen-treated mammary glands was about 35 and 80% of that seen in control mammary glands, respectively (Fig. 6*H*). Estradiol significantly increased both IRS-1 and IRS-2 as well as their basal tyrosyl phosphorylation ($P < 0.01$; Fig. 6, *F* and *H*).

Because the phosphorylated IGF-1R phosphorylates IRS-1 and IRS-2 and activates the signal Ras/Raf/MAPK pathways, the levels of c-Raf-1 and phospho MAPK were determined (19). As shown in Fig. 7, c-Raf-1 protein was decreased by 50% by ICI ($P < 0.01$). Estradiol increased c-Raf-1 by 2-fold ($P < 0.01$). Tamoxifen slightly increased c-Raf-1. Despite a significant reduction in levels of IGF-1R, IRS-1, IRS-2, and c-Raf-1, the levels of phospho p44/42 MAPK were slightly decreased by ICI and tamoxifen treatments. Basal phospho p44/42 MAPK was significantly low in estradiol-treated mammary tissue as compared with vehicle and antiestrogen-treated mammary glands ($P < 0.01$; Fig. 8).

Because PI-3K activity is important for IGF-1-induced mitogenesis and antiapoptosis (36, 37), the levels of the p85 subunit of PI-3K and its phosphorylation were investigated. Fig. 9 shows that the level of the p85 subunit of PI-3K did not change as a result of ICI and tamoxifen treatments. However, the basal phosphorylation of the p85 subunit of PI-3K was undetectable after ICI and tamoxifen treatments. Estradiol, on the other hand, significantly decreased the unphosphorylated p85 subunit of PI-3K and significantly increased basal phosphorylation of the p85 subunit of PI-3K ($P < 0.01$). One of the downstream effectors of PI-3K is the serine/threonine kinase Akt-1 (38), which was also decreased by ICI and increased by estradiol (Fig. 9, *C* and *E*).

DISCUSSION

Both tamoxifen and ICI influence expression of many genes involved in IGF signal transduction. The dominant effect is toward decreased signaling, which may be a consequence of

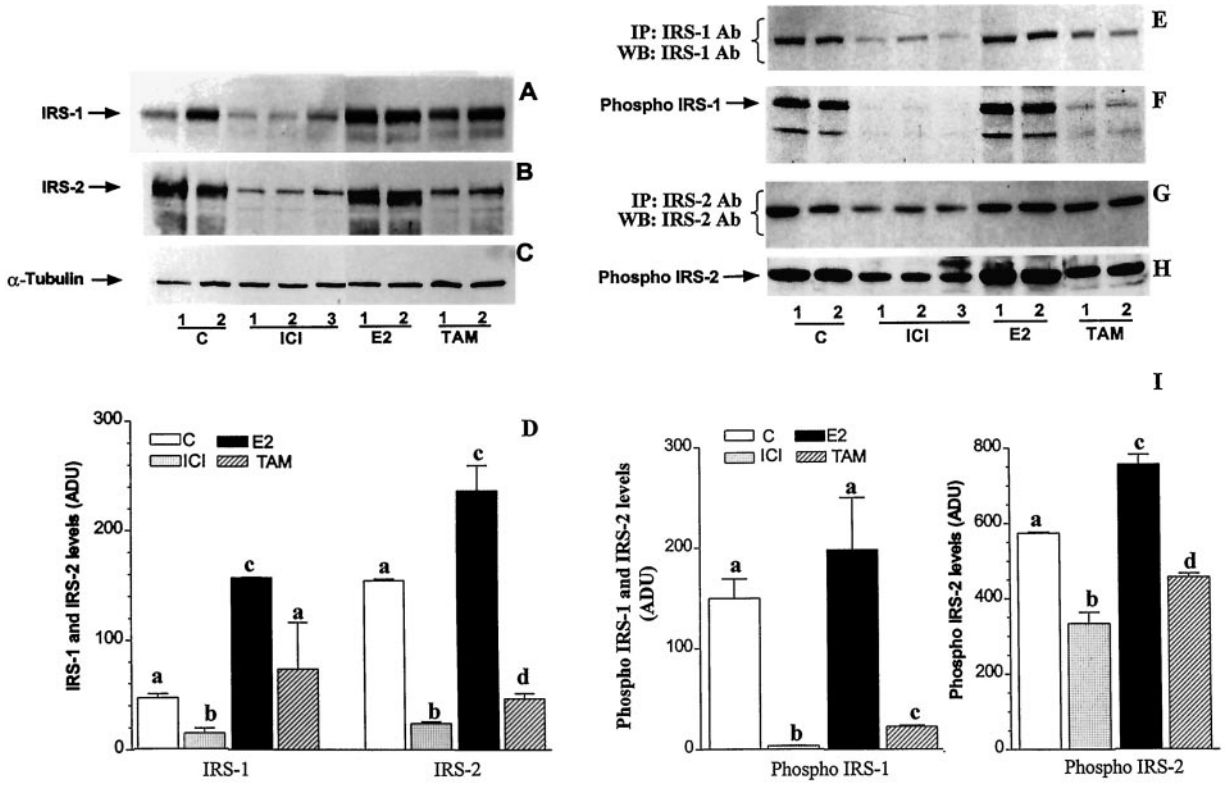

Fig. 6 Effects of ICI, tamoxifen (*TAM*), and estradiol (E_2) on IRS-1 and IRS-2 levels in the mammary gland. Rats were treated with the indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day), and ICI (2 mg/kg body weight/week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-IRS-1 (*A*), IRS-2 (*B*), and α-tubulin (*C*) antibodies. Densitometric scanning of the IRS-1 and IRS-2 is shown in *D*. Tissue lysates were immunoprecipitated with anti-IRS-1 (*E*) and anti-IRS-2 (*G*) antibodies. After SDS-PAGE, blots were incubated with anti-IRS-1 (*E*) and anti-IRS-2 (*G*) or antiphosphotyrosine antibodies (*F* and *H*). Densitometric scanning of tyrosine phosphorylated IRS-1 and IRS-2 is shown in (*I*). *Columns* with different *letters* are significantly different from one another; *bars*, SE. IRS-1 and IRS-2 levels were significantly decreased by ICI. Basal tyrosyl phosphorylation of IRS-1 was undetectable in ICI-treated mammary gland.

the large increase in *IGFBP-3* expression. The molecular mechanisms by which ICI increased IGF-1 mRNA in the mammary gland are unclear. The magnitude of ICI-induced *IGF-1* gene expression is far less than ICI-induced *IGFBP-3* gene expression. The antiproliferation activity of antiestrogens on breast epithelial cells are well correlated with their effects on *IGFBP-3* expression, assuming an IGF-dependent and -independent (16, 39, 40) growth-inhibitory action of this protein; the weaker inhibitory effect of tamoxifen is associated with mild stimulation of mammary *IGFBP-3* expression, whereas ICI-induced growth inhibition is associated with stronger up-regulation of mammary *IGFBP-3* expression (16).

The functional significance of IGFBP-2 and IGFBP-5 within the mammary gland and breast epithelial growth is unclear. *In vitro* both inhibition and potentiation of IGF activity by IGFBP-2 and IGFBP-5 have been reported (32). Our data demonstrate that *IGFBP-2* expression is stimulated by estrogen and inhibited by ICI and positively correlates with breast epithelial cell proliferation. It has been reported that *IGFBP-2* levels were elevated in serum from various cancer patients (13, 41–43), and overexpression of *IGFBP-2* resulted in increased tumorigenic potential of adrenocortical cells (44). It is possible that increased IGFBP-2 may potentiate the response to IGF-1 by breast epithelial cells. In the present study, *IGFBP-5* expression is inversely correlated with ICI-induced growth inhibition, which is different from previous reports (45) where *IGFBP-5* expression was greatly induced during mammary involution.

Evidence compatible with responsiveness of a large subset of breast cancers to exogenous IGFs includes studies documenting type 1 IGF receptor expression by primary human breast cancers and studies documenting *in vitro* and *in vivo* responsiveness of breast cancer to IGF-1 (4). Down-regulation of IGF-1R by 30–80% was sufficient to inhibit IGF-1-induced breast cancer cell proliferation (46). Treatment of experimental animals bearing IGF-1-responsive breast cancers with a blocking antibody directed against the type I IGF receptor resulted in significant reduction in the growth of the experimental neoplasms (47). The important role of IGF-1R in autocrine/paracrine activation of the IGF pathway in tumors (33), antiapoptosis (48, 49), and sensitivity to chemotherapy drugs (50) is well documented. These data indicate that IGF-1R is important for breast epithelial growth and suggest that the inhibition of IGF-1R expression may represent a strategy for treatment of breast cancer. *In vivo* down-regulation of IGF-1R and its basal autophosphorylation by ICI and tamoxifen (to a lesser extent) as we show here would expect to interfere with the IGF signal

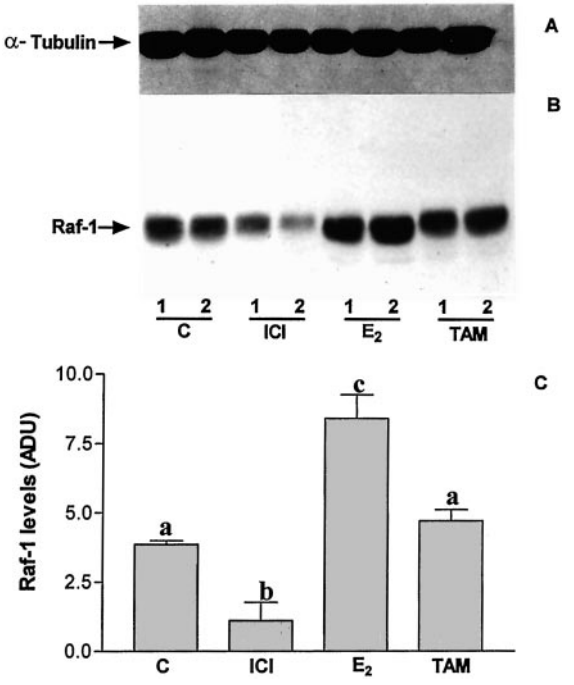

Fig. 7 Effects of ICI, tamoxifen (*TAM*), and estradiol (E_2) on c-Raf-1 levels in the mammary gland. Rats were treated with indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day), and ICI (2 mg/kg body weight/week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with anti-α-tubulin (*A*) and anti-c-Raf-1 (*B*) antibodies. Densitometric scanning of the c-Raf-1 is shown in *C*. *Columns* with different *letters* are significantly different from one another ($P < 0.01$); *bars*, SE. Estradiol significantly increases whereas ICI decreases c-Raf-1 levels.

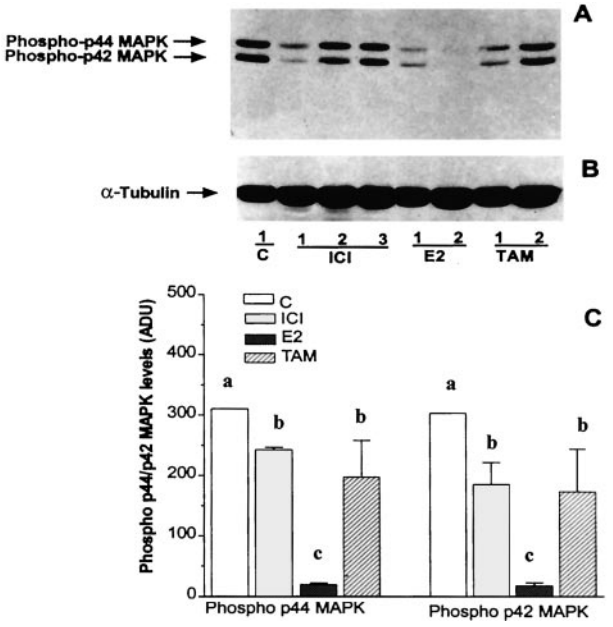

Fig. 8 Effects of ICI, tamoxifen (*TAM*), and estradiol (*E2*) on the levels of threonine/tyrosine phosphorylation of p42/44 MAPK in the mammary gland. Rats were treated with the indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day), and ICI (2 mg/kg body weight/week). Tissue lysates from mammary tissue were analyzed by Western blotting. Blots were incubated with phospho p44/42 MAPK (Thr-202/Tyr-204; *A*) and anti-α-tubulin (*B*) antibodies. Densitometric scanning of the phospho p44 and p42 MAPK is shown in *C*. *Columns* with different *letters* are significantly different from one another ($P < 0.01$); *bars*, SE. ICI slightly decreased phospho p44/42 MAPK whereas estradiol decreased phospho p44/42 MAPK levels.

cascade, leading to inhibition of cellular proliferation and enhancement of apoptosis.

In the present study, we observed high levels of threonine/tyrosine phosphorylation of MAPK after tamoxifen- and ICI-induced growth arrest. Furthermore, estradiol-induced proliferation was associated with reduction in basal threonine/tyrosine phosphorylation of MAPK. The observation that ICI increased threonine/tyrosine phosphorylation of MAPK was unexpected because autophosphorylation of IGF-1R and tyrosyl phosphorylation of IRS-1 and IRS-2 were significantly reduced after ICI treatment. The mechanisms by which ICI increases phospho MAPK are unclear. Although the nuclear translocation of MAPK has not been determined in our present study, it is possible that the duration of MAPK activation and nuclear translocation of the enzyme induced by ICI and estradiol may give rise to differences in the biological actions of ICI and estradiol on breast epithelial cell proliferation. The substrates of MAPK include transcription factors and other kinases (18, 51, 52). Phosphorylation of these transcription factors by MAPK may lead to induction of expression of new genes that are responsible for the antiproliferative effect. This hypothesis is supported by the observations that in PC12 cells, nerve growth factor-induced growth arrest was associated with sustained activation and nuclear translocation of MAPK whereas insulin- or EGF-induced proliferation was associated with transient activation of MAPK without pronounced nuclear translocation of the enzyme (52, 53).

Recent work into IGF-1 antiapoptosis signaling has demonstrated the importance of PI-3K and its downstream substrate Akt (54, 55). Akt was known to have a strong antiapoptotic effect in a wide range of cell types, mediating survival signals from many stimuli, particularly those that activate PI-3K including IGF-1. Furthermore, a direct link between PI-3K and the apoptosis-regulating Bcl family of proteins has been established through Akt phosphorylation of Bad (56, 57). Thus, the ability of ICI to reduce the p85 subunit of PI-3K and Akt-1 phosphorylation may be important for ICI-induced apoptosis. Experiments are under way to determine whether ICI also induces apoptosis of breast epithelial cells *in vivo*.

It has been reported that IGF-1 synergies along with estrogens to stimulate ductal morphogenesis (6) and treatment of mice with estradiol have no effect on mammary development in IGF-1 null mice (6). This observation may be attributable to the enhancement of IGF on the ligand-induced transcriptional activity of ER (58). The effects of estradiol on IGF-1R, IRS-1, IRS-2, PI-3K, and IGFBP-3 in the mammary tissue were consistent with previous reports showing that the effect of estrogen on cell growth was mediated by the up-regulation of IGF-1R expression (9), IRS-1, and IRS-2 (11) or by down-regulation of the inhibitory IGFBP-3 (16). The observations that ICI reduced

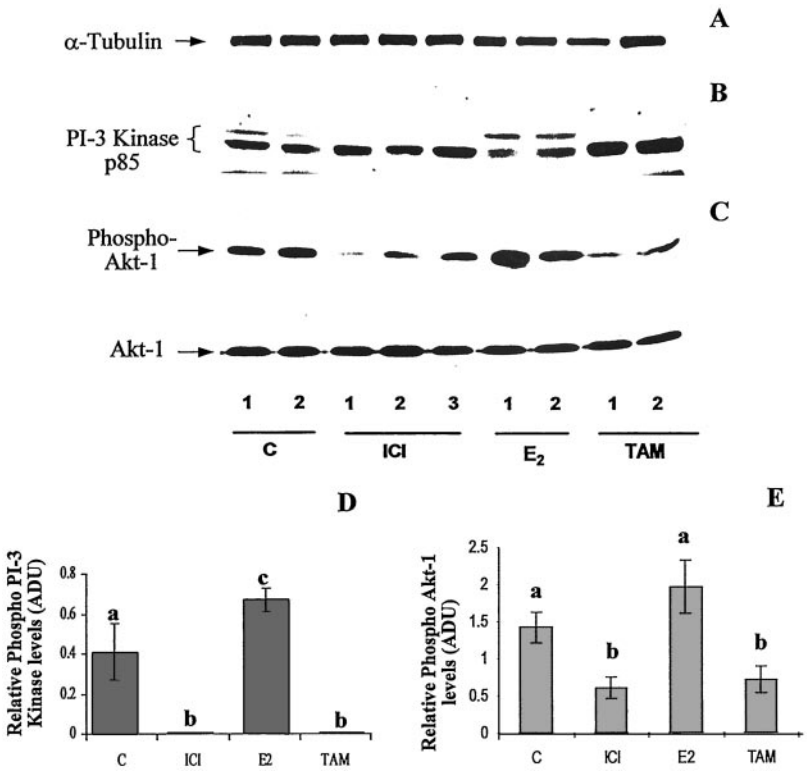

Fig. 9 Effects of ICI, tamoxifen (*TAM*), and estradiol (E_2) on the levels of p85 subunit of PI-3K in the mammary gland. Rats were treated with the indicated concentrations of estradiol (1.2 μg/day), tamoxifen (100 μg/day), and ICI (2 mg/kg body weight/week). Tissue lysates from mammary tissue were analyzed by Western blotting as described in "Materials and Methods." Blots were incubated with anti-α-tubulin (*A*) and anti-p85 subunit of PI-3K (*B*), and anti-phospho Akt-1 antibodies (*C*). Densitometric scanning of the phospho p85 subunit of PI-3 kinase (*D*) and phospho Akt-1 (*E*) are shown. *Columns* with different *letters* are significantly different from one another ($P < 0.01$); *bars*, SE. Estradiol significantly induces phosphorylation of the p85 subunit of PI-3K and Akt-1 phosphorylation. ICI reduced the basal p85 subunit of PI-3K phosphorylation and phospho Akt-1 levels without affecting the levels of the p85 subunit of PI-3K levels.

basal phosphorylation of IGF-1R, IRS-1, IRS-2, Akt-1, and the p85 subunit of PI-3K in the mammary gland indicate that *in vivo* inhibition of breast epithelial cell growth by ICI may not only be attributable to competition between estrogens and ICI for ER but also interruption of IGF signaling pathway. By doing so, ICI may also block a possible cross-talk between the ER and IGF-1R signaling pathways (12).

Our data provide evidence for an *in vivo* action of antiestrogens. The antiproliferative effect of antiestrogens is associated with altered expression of several proteins involved in the IGF signal pathway and their phosphorylation. Our *in vivo* results confirm and extend prior works that demonstrated enhancement of IRS-1 expression in ER-positive breast cancer cells by estrogens (11). Furthermore, IRS-1 (59) and the IGF-1R signaling pathway (60) are the targets for ICI 182,780 and tamoxifen, respectively, in breast cancer cells.

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