Regulation of Insulin-like Growth Factor I Receptor Expression by the Pure Antiestrogen ICI 182780[1]

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ABSTRACT

Insulin-like growth factor I (IGF-I) is a mitogen for human breast cancer cells both *in vivo* and *in vitro*. We demonstrate here that the antiestrogen ICI 182780 (ICI) at 10^{-8} M decreases IGF-I receptor (IGF-IR) mRNA levels by 70% after treatment for 48 h. Measurements of mRNA stability indicate that the half-life of IGF-IR mRNA is approximately 3 h. Estradiol treatment increases the half-life of the IGF-IR mRNA to approximately 6 h and the level of *IGF-IR* gene transcription by 1.8-fold, whereas ICI treatment not only decreases the IGF-IR transcription rate by 50% but also decreases the IGF-IR mRNA half-life to less than 3 h. Affinity labeling studies with [^{125}I]-IGF-I show 35% increased labeled IGF-I to MCF-7 cell membrane following estradiol treatment and 40% decreased labeling following ICI treatment. We also demonstrate that ICI attenuates IGF-I-stimulated growth. Our data suggest that attenuation of IGF-I responsivity by ICI may be due in part to reducing the IGF-IR expression.

INTRODUCTION

The IGF-IR[3] is a tyrosine kinase receptor with 70% homology to the insulin receptor (1) and is activated by IGF-I and IGF-II (2). The IGF-IR is expressed in many different tumor types (3) and overexpressed in some, including human cervical cancer (4). The importance of IGF-IR in tumor growth has recently been reviewed (5). IGF-IR is required for transformation by SV40 large T antigen (6), for growth (7, 8), for the establishment and maintenance of a transformed phenotype for several cell types (7, 9–11), for transducing mitogenic signals initiated by other growth factors, such as epidermal growth factor and platelet-derived growth factor (10), and for protecting tumor cells from apoptosis both *in vitro* and *in vivo* (12).

Overexpression of the IGF-IR is capable of promoting ligand-dependent neoplastic transformation in several cell types (13). Overexpression of both IGF-IR and IGF-I in cells can bypass the requirements of exogenous growth factors for growth (14).

It has been shown that the IGF-IR is up-regulated in ER-positive MCF-7 human breast cancer cells in response to estradiol (15). This report suggests that estrogen sensitizes MCF-7 to the mitogenic effects of IGFs by up-regulation of the IGF-IR. Previous reports based on experiments with other antiestrogens showed up-regulation (16), no change, or down-regulation (17) of IGF-IR levels, but none of these examined the transcription rate of the *IGF-IR* gene or IGF-IR mRNA half-life. We conducted experiments to study the effect of complete antiestrogen ICI (18) and estradiol on IGF-IR expression.

MATERIALS AND METHODS

Cell Culture and Thymidine Incorporation. Human breast cancer MCF-7 cells were maintained as monolayer cultures as described previously (19). For proliferation experiments, stock cultures were trypsinized and plated at 2.5×10^4 cells/well in 24-well dishes in PF α-MEM supplemented with 2.5% fetal bovine serum. After 48 h, cells were withdrawn from estrogens by culturing for another 24 h in PF α-MEM supplemented with 2.5% double charcoal-stripped fetal bovine serum. Cells were then washed twice with serum-free α-MEM followed by a 3-h incubation in serum-free PF-α-MEM media. Treatments were given in serum-free PF-α-MEM for 48 h. Thymidine incorporation was measured as described (20). Experiments were carried out in triplicate and repeated at least three times. For RNA, nuclear isolation, and membrane extraction, cells were treated as described above, except they were initially plated at 5×10^6 cells in 100-mm dishes.

Northern Blot Analysis. RNA was extracted from cells using the RNAZolB method (Tel-Test, Friendswood, TX). Total RNA was fractionated on 1% agarose gels and filters were prepared as described previously (21). Human IGF-IR cDNA (American Type Culture Collection) was labeled with [α-^{32}P]dCTP using a T7 Quick-Prime kit (Pharmacia). The blots were subjected to autoradiography for 1 to 3 days with an intensifying screen at −80°C. Equal loading of RNA across lanes was controlled for by rehybridizing the blots with labeled rat 18S rRNA cDNA (American Type Culture Collection). Quantitative analysis of gene expression was accomplished by scanning autoradiographs densitometrically. For determination of the mRNA half-life, transcription of cellular mRNA was inhibited by treating cells in parallel with 25 μg/ml DRFB as described (22). Total RNA was extracted from cells every 3 h after DRFB treatment and analyzed with Northern blotting using IGF-IR cDNA. These blots were rehybridized with human β-actin cDNA probe as a control for inhibition of transcription as described previously (22).
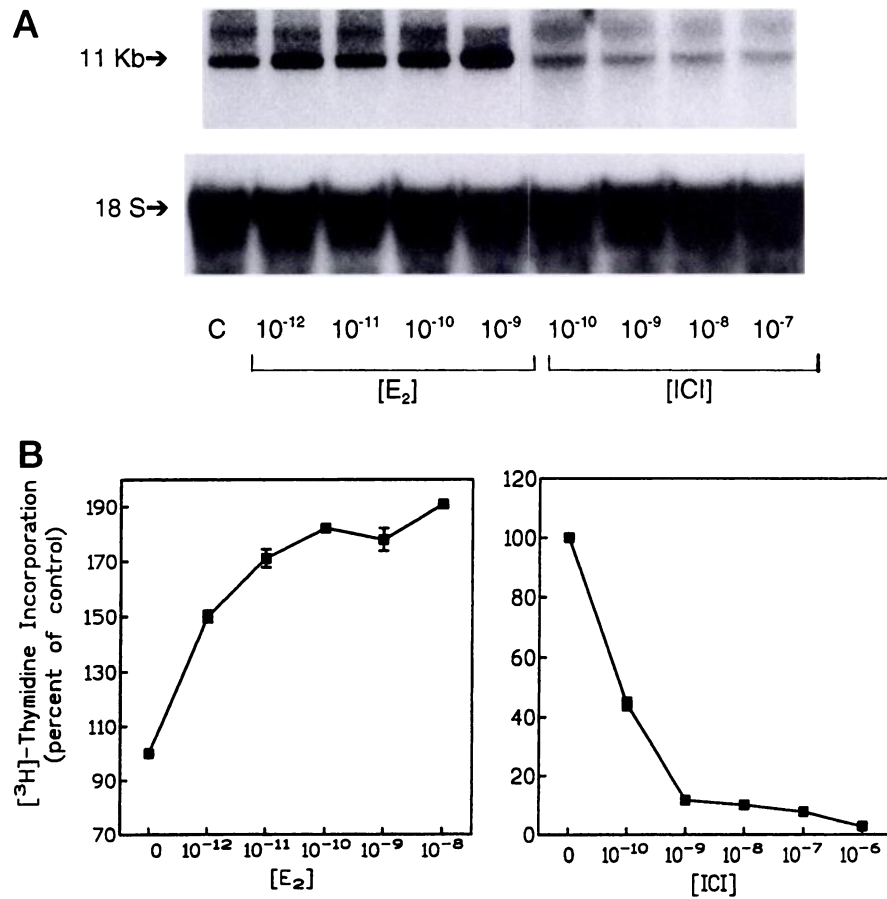
A

11 Kb→

18 S→

C 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-10} 10^{-9} 10^{-8} 10^{-7}

[E₂]   [ICI]

B

[³H]-Thymidine Incorporation (percent of control)

190
170
150
130
110
90
70

0 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8}

[E₂]

120
100
80
60
40
20
0

0 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

[ICI]

Fig. 1 Regulation of IGF-IR mRNA levels by estradiol and ICI. MCF-7 cells were grown in PF containing 2.5% fetal bovine serum. Cells were withdrawn from residual estrogens in serum by culturing for an additional 24 h in PF-α-MEM supplemented with 2.5% double charcoal-stripped fetal bovine serum. Cells were then washed twice with serum-free α-MEM followed by a 3-h incubation in serum-free media. Cells were treated with the indicated doses of estradiol or ICI in PFSF medium for 48 h. Cytoplasmic RNA was isolated and Northern blotting was performed as described in "Materials and Methods." Densitometric scanning of the autoradiographs was used to quantitate IGF-IR mRNA levels. *A*, the 11-kb IGF-IR mRNA (*top*) and 18S rRNA (*bottom*) are shown. The experiments were repeated at least three times with similar results. *B*, proliferation of MCF-7 cells in various concentrations of estradiol and ICI in PFSF media was estimated by [³H]thymidine incorporation. Experiments were performed in triplicate and repeated three times. The results are expressed as the average of three experiments. *Bars*, SE.

Nuclear Run-Off Assay. MCF-7 cells were grown in PF medium supplemented with 2.5% double charcoal-stripped fetal bovine serum for 24 h. Cells were washed with PFSF medium three times followed by a 3-h incubation period in PFSF. Cells were treated with either 10^{-10} M estradiol or 10^{-7} M ICI for 48 h in serum PFSF medium. Nuclei were isolated and nuclear run-off assays for *IGF-IR* and β-actin genes were performed as described previously (23).

IGF-IR Binding Assay. Cell membrane extracts were prepared from MCF-7 cell monolayers. Cells were washed with ice-cold PBS and solubilized in 6 ml sodium bicarbonate-EDTA buffer (1 mM NaHCO₃ and 1 mM EDTA). Following homogenization, cells were centrifuged at 4°C for 10 min at 3500 rpm to pellet the nuclei. To pellet the cellular membrane, the supernatant was centrifuged at 14,000 rpm for 45 min at 4°C. Membrane extracts containing 100 μg of membrane protein were incubated with 500,000 cpm ¹²⁵I-IGF-I in PBS containing 1% BSA overnight at 4°C. Ligand was then cross-linked to recep-

tors by incubation with 0.8 ml of 0.3 mM DSS for 1 h at 4°C followed by quenching with 200 μl of 1 M Tris (pH 6.8). The membrane was pelleted as before and radioactivity was measured in a gamma counter. Proteins were separated by SDS-PAGE under reducing conditions followed by transfer to nitrocellulose membrane and autoradiography.

RESULTS

Effects of Estradiol and ICI on *IGF-IR* Gene Expression and Cell Proliferation. Treatment of MCF-7 cells for 48 h with a range of concentrations of estradiol resulted in a dose-dependent increase in IGF-IR mRNA abundance (Fig. 1A). The IGF-IR mRNA levels were increased by 2.5-fold when the cells were exposed to 10^{-10} M estradiol, consistent with reports by Stewart *et al.* (15). In contrast, treatment with ICI resulted in dose-dependent decrease in IGF-IR mRNA. IGF-IR mRNA levels were decreased by 70 ± 7.6% at a dose of 10^{-8} M ICI,
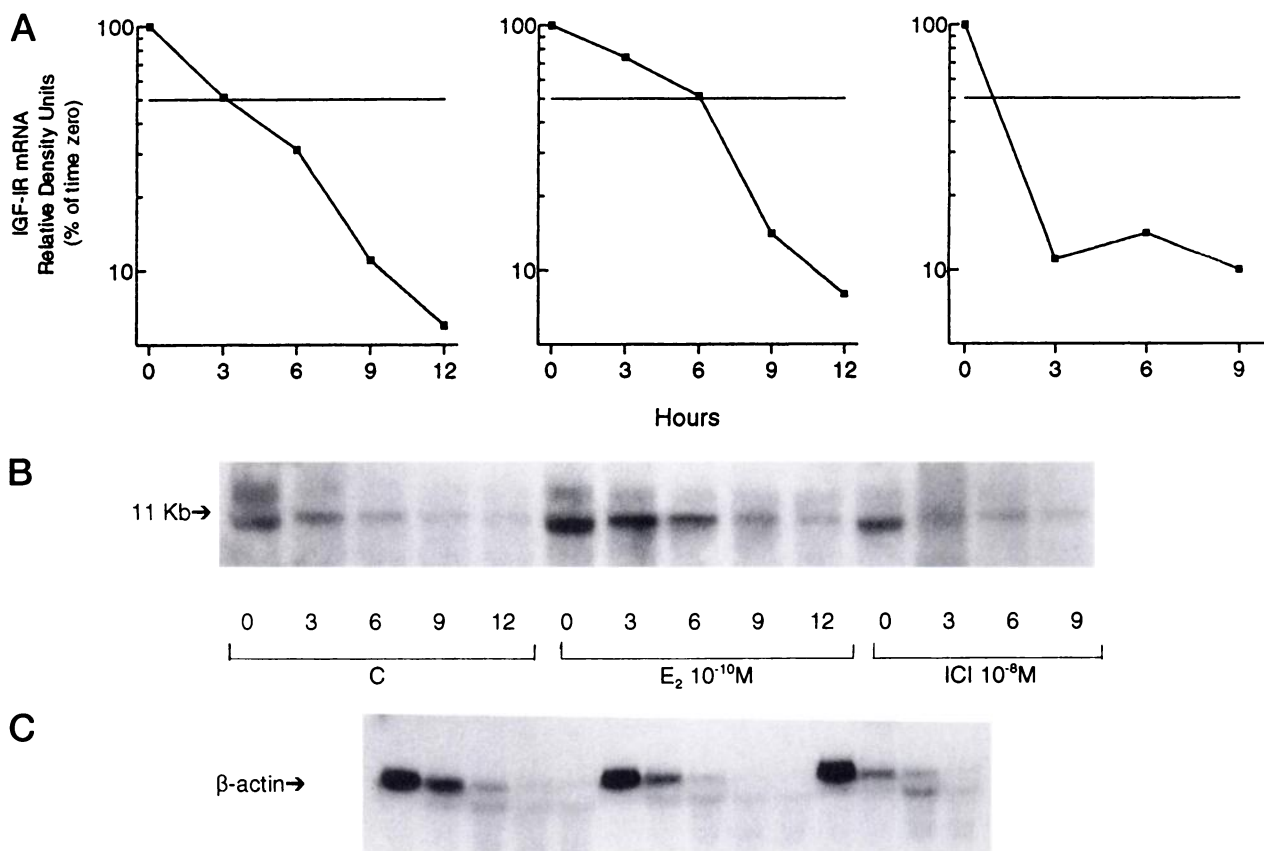
Fig. 2   Half-life of IGF-IR mRNA in response to estradiol or ICI treatment. MCF-7 cells were grown and treated with the indicated doses of estradiol and ICI as described in the legend to Fig. 1. The half-life of IGF-IR mRNA (B) and β-actin mRNA (C) were determined as described in "Materials and Methods." The experiments were repeated three times with similar results. The half-life of IGF-IR mRNA was estimated by densitometric scanning of Northern blots (A). The half-life of control β-actin mRNA was determined to be approximately 3 h, which is in agreement with a previous study (22).

and the same dose decreased cell proliferation by 80% (Fig. 1B). To confirm that the inhibitory effect of ICI is specific in our experimental system, rather than a result of opposing the action of residual estradiol, we probed the Northern blots with pS2 cDNA which is known to be stimulated by estradiol in MCF-7 cells (24, 25). It was found that the pS2 levels were not suppressed by treatment with ICI, suggesting that the estrogen-stripping protocol was adequate (data not shown).

Effects of Estradiol and ICI on IGF-IR mRNA Half-Life.   To further examine the mode of IGF-IR mRNA regulation by estradiol and ICI, the IGF-IR mRNA stability was estimated using the mRNA-specific transcription inhibitor DRFB. As shown in Fig. 2, the half-life of mRNA in control cells was approximately 3 h. This half-life was increased to about 6 h in cells treated with 10^{-10} M estradiol and decreased to less than 3 h when treated with 10^{-8} M ICI in estrogen-free conditions. The half-life for β-actin mRNA was approximately 3 h under all conditions, in agreement with a previous study (22).

Effects of Estradiol and ICI on the Transcription Rate of the *IGF-IR* Gene.   To determine whether ICI or estradiol also modulates the transcription rate of the *IGF-IR* gene, nuclear

run-off experiments were performed. As shown in Fig. 3, the level of *IGF-IR* gene transcription was decreased by 50% when cells were exposed to ICI and increased by 1.8-fold in the presence of estradiol.

Effects of Estradiol and ICI on Binding of ^{125}I-IGF-I to the IGF-IR.   Estradiol increased the binding capacity of MCF-7 cell membranes for ^{125}I-IGF-I (Fig. 4). Treatment with 10^{-10} M estradiol resulted in a 35 ± 5.4% increase in binding. This difference was significant ($P < 0.05$) as determined by Student's t test. Treatment with ICI resulted in a dose-dependent decrease in binding to the receptor (Fig. 4). Treatment with 10^{-8} M ICI decreased binding by 40 ± 3.8% compared to control.

Effects of ICI on IGF-I-stimulated Growth.   Responsivity of MCF-7 to the mitogenic effects of IGF-I was attenuated in the presence of 10^{-9} M ICI (Fig. 5A). Ten ng/ml (1.4×10^{-9} M) IGF-I increased baseline DNA synthesis by MCF-7 cells 2.5-fold, and this stimulation was completely blocked by 10^{-9} M ICI. Treatment with increasing concentrations of ICI in the presence of 10 ng/ml IGF-I resulted in dose-dependent inhibition of IGF-I-stimulated growth (Fig. 5B).
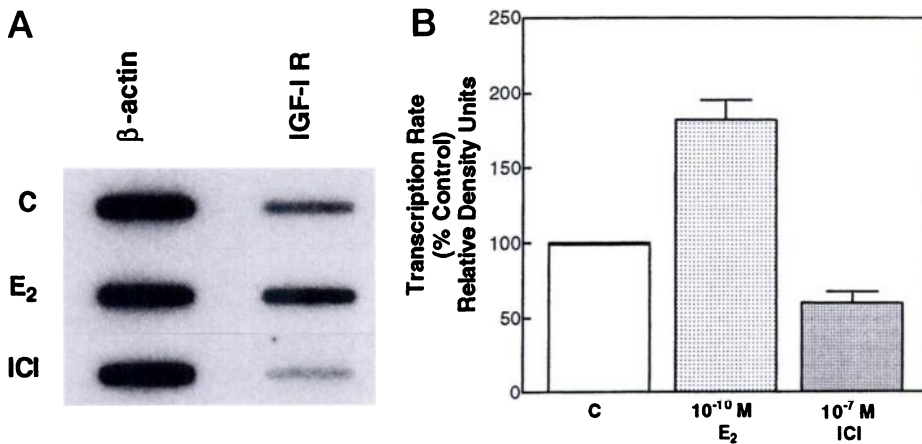
A

β-actin   IGF-I R

C

E₂

ICI

B

Fig. 3 Effects of estradiol and ICI on the transcription rate of the *IGF-IR* gene. MCF-7 cells were grown as described in the legend to Fig. 1. Cells were treated with either PFSF as control (C) or 10^{-10} M estradiol (E_2) or 10^{-7} M ICI in PFSF medium for 48 h. Nuclear run-off assays were conducted as described previously (23). Representative nuclear run-off results are shown in *A*, and densitometric quantitation is shown in *B*. The experiments were repeated twice with similar results. *Bars*, SE.

A

200 KDa→

130 KDa→

0   10^{-12} 10^{-11} 10^{-10} 10^{-9} 0 10^{-10} 10^{-9} 10^{-8} 10^{-7}

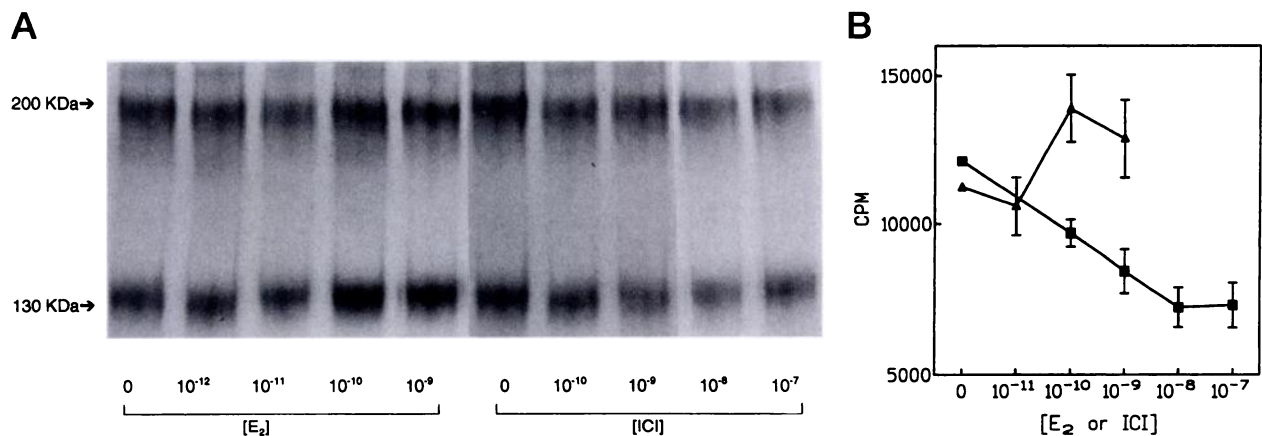[E₂]   [ICI]

B

Fig. 4 Effects of estradiol and ICI on binding of ^{125}I-IGF-I to the IGF-IR. MCF-7 cells were grown as described in the legend to Fig. 1. Cells were treated with various doses of estradiol or ICI for 48 h. *A*, a representative autoradiograph of the IGF-IR affinity labeling. Membrane extracts were prepared and incubated with ^{125}I-IGF-I. Following cross-linking with DSS, the cross-linked protein complexes were analyzed with SDS-PAGE under reducing conditions as described in "Materials and Methods." *B*, prior to gel electrophoresis, total ^{125}I-IGF-I bound to membrane extracts from cells treated with estradiol (▲) or ICI (■) was determined by measuring radioactivity. Molar concentrations are indicated. *Bars*, SE.

DISCUSSION

Our data suggest that ICI reduces the *IGF-IR* gene transcription rate and IGF-IR mRNA half-life and cell surface IGF-IR levels in MCF-7 cells. We also show that ICI reduces responsivity of these cells to the proliferative effects of IGF-I. These results are in keeping with previous work where estradiol increases IGF-IR expression (15). The increased mitogenic responsivity to IGFs in the presence as opposed to the absence of estrogens can be explained in part by enhancement of the growth factor-Ras-Raf-MAPK cascade described by Kato *et al.* (26). Similarly, tamoxifen was found to up-regulate the IGF-IR in tamoxifen-resistant RL-3 cells but not in MCF-7 parental cells, suggesting that tamoxifen stimulates cell proliferation in RL-3 cells in part by sensitizing cells to the proliferative effects of IGF-I (16).

Our data suggest that the IGF-IR in breast cancer cells is down-regulated by a pure antiestrogen and extends a previous report in which surface receptor for IGF-I was reduced in MCF-7 in response to 4-hydroxytamoxifen treatment (17).

Reduction of the IGF-IR expression observed in the present studies is due in part to the decrease in both the transcription rate and mRNA half-life.

ICI is a potent inhibitor of MCF-7 cell proliferation (18, 19). Attenuation of IGF-I-stimulated growth by ICI is in part due to the reduction in IGF-IR expression and probably through estrogen-signaling pathways and growth factor-signaling pathways (26). Since IGF-I activates MAPK (26), the reduction in IGF-IR reported here and increase in IGF-binding proteins (19, 27) by ICI would result not only in a reduction cellular responsivity to IGFs but also MAPK activity. Since phosphorylation of ER by MAPK is essential for transcriptional activity of ER (26), which in turn induces an IGF-II autocrine loop (28), reduction in MAPK activity by ICI-induced decreased IGF-IR expression could also reduce transcriptional activity of ER, thereby reducing the cellular responsivity to both estrogen and IGFs. However, the change in IGF-IR alone cannot account for a major portion of the inhibitory action of ICI, since the magnitude of the decline in the IGF-IR level is small relative to the
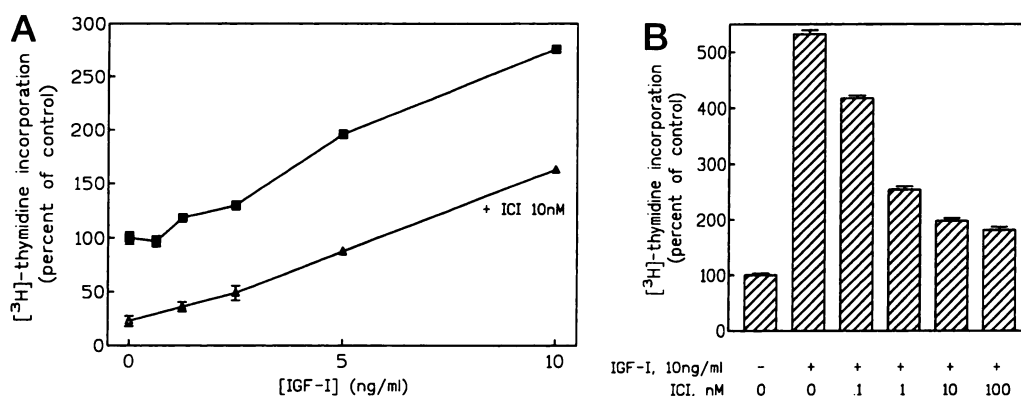
Fig. 5 Effects of ICI on IGF-I-stimulated proliferation in MCF-7. MCF-7 cells were grown and treated as described in "Materials and Methods." Proliferation of cells in various concentrations of IGF-I alone (■) or in the presence of 10^{-8} M ICI (▲, *A*) or in varying doses of ICI in the presence of 10 ng/ml IGF-I (*B*) was determined by [^{3}H]thymidine incorporation. The experiments were repeated four times with similar results. The results represent the mean of four experiments. *Bars*, SE.

pronounced inhibitory effect on proliferation. Other mechanisms by which ICI can reduce IGF responsivity have also been proposed (19, 27).

The IGF-IR has been implicated as an important determinant of malignant proliferation not only as a mitogen but also as an inhibitor of apoptosis (12, 29). The ability of MCF-7 cells to undergo apoptosis *in vitro* in response to ICI remains controversial (30). Our data raise the possibility that induction of apoptosis in MCF-7 cells by ICI may be related in part to the reduction of IGF-IR levels.

Our results provide another example of the effect of antiestrogens on the IGF system of mitogens. At the whole organism level, tamoxifen has been shown to decrease growth hormone output by the pituitary gland (31), decrease the serum IGF-I level (32), and decrease *IGF-I* gene expression (33). At the cellular level, ICI not only modulates IGF binding protein secretion (19) but, as shown here, also inhibits IGF-IR expression. All of these actions tend to decrease IGF bioactivity and thus may contribute to the therapeutic effect of antiestrogens.

ACKNOWLEDGMENTS

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