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# Interleukin-1 Blocks Insulin and Insulin-Like Growth Factor-Stimulated Growth in MCF-7 Human Breast Cancer Cells by Inhibiting Receptor Tyrosine Kinase Activity*

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**ABSTRACT**

Interleukins-1 (IL-1s) are known to inhibit the growth of cultured breast cancer cells. We examined the effects of IL-1$\alpha$ and IL-1$\beta$ on insulin and insulin-like growth factor I (IGF-I) stimulation of cell growth and found that both IL-1s inhibited anchorage-dependent and independent growth of MCF-7 breast cancer cells. In cells incubated with IL-1$\beta$ (100 U/ml), insulin receptor (IR) protein and messenger RNA were increased by 100%, while IGF-I receptor protein and transcript were not significantly changed. These data were confirmed by binding studies. Incubation of MCF-7 cells with IL-1s led, however, to a significant inhibition of IR and IGF-I receptor autophosphorylation (−55%) and phosphotransferase activity (−65%). Also, in 3T3/HIR rat fibroblasts, transfected with and overexpressing IR, IL-1s decreased insulin-stimulated cell growth in soft agar and IR tyrosine kinase activity.

The present findings suggest that IL-1s antagonize the insulin and IGF-I mitogenic effects in MCF-7 cells by blocking the receptor tyrosine kinase activity that is crucial for the mitogenic effect of these factors. (*Endocrinology* **137:** 4100–4107, 1996)

INTERLEUKINS-1 (IL-1$\alpha$ and IL-1$\beta$) are two related polypeptides produced mainly by activated macrophages. Although they have only 26% homology, ILs bind to common receptors and elicit a variety of biological responses, including the modulation of immunological, inflammatory, and host defense mechanisms (1–4). They also have antineoplastic effects, decreasing tumor growth both by a direct cytostatic and cytotoxic action (5, 6) and also by the indirect mechanism of stimulating the host immune response (7, 8). Because of these properties, IL-1s are under clinical trials for the treatment of a variety of malignancies (9).

IL-1s have been shown to inhibit the growth of certain, but not all, breast cancer cell lines (10–12). In the estrogen receptor-positive human breast cancer cells MCF-7, ZR-75, and T47-D, IL-1$\alpha$ antagonizes estradiol stimulation of growth and reduces estrogen receptor content (13). It is unlikely, however, that the estrogen receptor down-regulation induced by IL-1 is responsible for the decreased mitogenic responsiveness to estradiol. In fact, in these cells, estrogen receptor molecules are present in considerable excess, and the inhibitory effect of IL-1$\alpha$ is restricted to cell growth and is not observed for other estradiol-regulated processes (13).

The growth of breast cancer cells is regulated by a variety of peptide hormones and growth factors in addition to estrogens. In particular, insulin and insulin-like growth factor I (IGF-I) receptors are expressed at high levels in most breast cancer cells (14, 15); both are required for their optimal growth (16, 17) and exert a potent mitogenic effect. Interestingly, IL-1 inhibits proliferation in breast cancer cells that respond to insulin and IGF-I, whereas it is without effect on the growth of MDA-MB231 breast cancer cells (8, 10), which are insensitive to the mitogenic effect of insulin and IGF-I (18). These observations support a possible relationship between the antimitogenic effect of IL-1 and insulin/IGF-I-stimulated growth in breast cancer cells.

Accordingly, in the present study, we investigated whether IL-1s affected the growth response to insulin or IGF-I as well as insulin or IGF-I receptor (IGF-I-R) content and function in breast cancer MCF-7 cells. We found that IL-1s at low concentrations markedly antagonize the mitogenic effect of both insulin and IGF-I by inhibiting receptor tyrosine kinase activity.

## Materials and Methods

The following materials were purchased: MEM, FCS, glutamine, and gentamicin from Life Technologies (Paisley, UK); *N*-acetyl-D-glucosamine, BSA (RIA grade), bacitracin, phenylmethylsulfonylfluoride (PMSF), transforming growth factor-$\beta$ (TGF$\beta$), indomethacin, poly-Glu$^4$:Tyr$^1$ (PGT), and porcine insulin from Sigma Chemical Co. (St. Louis, MO); wheat germ agglutinin-agarose (WGA) from Miles Scientific (Naperville, IL); human recombinant IL-1$\alpha$ and IL-1$\beta$ (SA, $>5 \times 10^8$ U/mg), and [$\gamma$-$^{32}$P]ATP (111 terabecquerels/mmol) from Amersham International (Aylesbury, UK); and protein A-Sepharose from Pharmacia Biotech (Uppsala, Sweden).

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$Tyr_{A14}$-$^{125}$I-labeled insulin (SA, 13.3 megabecquerels/$\mu$g) was kindly provided by Dr. R. Navalesi (Pisa, Italy).

### *Cells*

MCF-7 human breast cancer cells were provided by Dr. I. Perroteau (Turin, Italy). They were grown in MEM supplemented with 7% FCS, 2 mM glutamine, nonessential amino acids, and 40 $\mu$g/ml gentamicin. NIH-3T3 cells transfected with the insulin receptor (IR) complementary DNA (cDNA) and overexpressing IRs (3T3/HIR; 631 $\pm$ 16.7 ng IR/$10^6$ cells) were provided by Dr. Whittaker (19, 20). They were grown in DMEM supplemented with 7% FCS, 2 mM glutamine, and 40 $\mu$g/ml gentamicin. The medium was routinely changed every 2 days.

### *Cell growth experiments in monolayer cultures*

Cells were seeded in MEM containing 7% FCS and 2 mM glutamine in 12-multiwell plates at a density of $1 \times 10^5$ cells/dish. Subconfluent cell monolayers were washed twice with 1 ml serum-free medium and transferred to 2 ml fresh serum-free medium consisting of MEM without phenol red containing 0.1% BSA. After 24 h, the medium was changed, and stimulators were added at the indicated concentrations. Medium was then changed every other day. At the end of a 5-day period, the cells were harvested with $Ca^{2+}$- and $Mg^{2+}$-free PBS-1 mM EDTA and counted on a hemocytometer. Cells were then centrifuged, and the DNA content of the cellular pellet was determined by the method of Labarca and Paigen (21).

### *Colony formation in soft agar*

To test the effect of IL-1$\beta$ on the ability of cells to form colonies in soft agar, subconfluent cell monolayers were detached with PBS containing 0.02 mM EDTA and 2 mg/ml trypsin. Cells were centrifuged, resuspended in the medium without phenol red to obtain single cell suspensions, and seeded in 35-mm petri dishes ($2 \times 10^4$ cells/dish) together with an equal volume of prewarmed (42 C) 0.66% bacto-agar suspension. The final concentration of agar was 0.33%. The effects of insulin, IGF-I, and IL-1$\beta$ on colony formation in soft agar were tested by adding these substances dissolved in 20 $\mu$l medium, every 24 h. The number and size of colonies were scored at inverted microscope 1 and 10 days after plating (20).

### *Insulin and IGF-I receptor protein and gene expression*

*[$^{125}$I]Insulin and [$^{125}$I]IGF-I binding to intact cells.* [$^{125}$I]Insulin and [$^{125}$I]IGF-I binding studies were carried out in intact cells grown to 80% confluent monolayers. Cells were plated in 35-mm multiwell plates at a concentration of $3 \times 10^5$ cells/well. After 48 h, the medium was replaced with MEM without phenol red containing 0.25% BSA and 0.5% dextran-coated charcoal-stripped FCS with or without IL-1s. At the end of the incubation period the cell monolayer was washed twice with PBS and incubated for 16 h at 4 C with [$^{125}$I]insulin or [$^{125}$I]IGF-I (40 pM) in 1 ml binding buffer, pH 7.8 (50 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 15 mM $NaC_2H_3O_2$, 10 mM glucose, 1 mM EDTA, and 10 mg/ml BSA), without or with increasing concentrations of unlabeled insulin or IGF-I. At the end of incubation, cell monolayers were washed with 1 ml binding buffer at 4 C, then solubilized with 0.03% SDS, and radioactivity was counted in a $\gamma$-counter.

Binding was corrected for nonspecific binding determined in the presence of an excess of unlabeled insulin or IGF-I (<5% of the total binding). Scatchard plots were calculated using the computer software Ligand.

*IR and IGF-I-R RIA.* Cell extracts for receptor RIA were prepared from cell monolayers preincubated or not with various doses of IL-1$\beta$ for the indicated periods. Cells were harvested with PBS containing 0.2% EDTA and counted in a Neubauer chamber. Typically, approximately $3 \times 10^6$ cells were centrifuged for 10 min at $400 \times g$; the pellet was resuspended in 1 ml 50 mM HEPES buffer, pH 7.4, containing 1 mg/ml bacitracin, 1 mM PMSF, and 1% Triton X-100 and solubilized for 60 min at 4 C. The solubilized material was then centrifuged at $10{,}000 \times g$, and the supernatant was frozen at $-80$ C until assayed. The cellular DNA content was measured by the method of Labarca and Paigen (21), and protein content in the cellular extracts was measured by the BCA method (Pierce Chemical Co., Rockford, IL). Insulin and IGF-I receptor RIAs were carried out as previously described (15, 22).

*RNA analysis.* Polyadenylated [poly(A)$^+$] RNA was extracted from cell monolayers (typically $10^8$ cells) using a one-step method as previously described (23). Briefly, adherent cells were released with proteinase K (final concentration, 0.3 mg/ml) and solubilized in 1% SDS. Oligo(deoxythymidine)-cellulose was directly added to the lysate and incubated overnight at 22 C. Poly(A)$^+$ RNA was eluted from oligo(deoxythymidine)-cellulose by adding 3 ml 10 mM Tris with 0.1 mM EDTA and 0.2% SDS.

Slot blot analysis was carried out using two human IR cDNA probes, 18.2 and 13.2 (1 and 4.2 kilobases, respectively), a kind gift from Dr. G. I. Bell (University of Chicago, Chicago, IL). A probe for the 36 B4 gene (24), kindly provided by Dr. P. Chambon (Strasbourg, France), was used as a control. cDNA probes were labeled with [$\gamma$-$^{32}$P]CTP using random primers to a specific activity of $10^9$ cpm/$\mu$g. The nitrocellulose filters from slot blots were prehybridized, hybridized, and washed as previously described (14). The intensity of signals was quantitated by scanning densitometry, and IR and IGF-IR messenger RNAs (mRNAs) were normalized using 36 B4 as a reference mRNA.

### *IR and IGF-I-R kinase studies*

*Receptor autophosphorylation.* IR and IGF-I-R $\beta$-subunit autophosphorylation was measured by a sensitive enzyme-linked immunoabsorbent assay. Briefly, serum-starved cell monolayers were incubated in the presence or absence of IL-1s. Either insulin or IGF-I was then added at the indicated concentrations for 10 min. The cells were solubilized, and receptors were captured by incubating cell lysates in Maxisorb plates (Nunc, Roskilde, Denmark) precoated with MA-20 (a monoclonal antibody specific to the IR) or $\alpha$IR-3 (a monoclonal antibody specific to the IGF-I-R). After washing, the phosphorylated receptors were incubated with a biotinylated antiphosphotyrosine antibody (Transduction Laboratories, Lexington, KY; 0.3 mg/ml in 50 mM HEPES-buffered saline, pH 7.6, containing 0.05% Tween-20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin, and 1 mM PMSF) and then with peroxidase-conjugated streptavidin. The peroxidase activity was determined colorimetrically by adding 100 $\mu$l *o*-phenylenediamine (0.67 mg/ml in 0.1 M citrate-phosphate buffer, pH 5.0, with 0.4 $\mu$l/ml 30% $H_2O_2$) and measuring the absorbance at 490 nM.

*Phosphotransferase activity measurement.* Phosphotransferase activity was measured in solubilized receptors from cells incubated in the presence or absence of IL-1s. Subconfluent 100-mm plates of cells preincubated or not with IL-1s for 48 h were harvested and solubilized for 60 min at 4 C in a buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 2 mM PMSF. Cell glycoproteins were obtained by WGA chromatography and eluted with 50 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 0.2 mM PMSF, and 0.3 M *N*-acetyl-D-glucosamine.

To measure receptor phosphotransferase activity, WGA-purified glycoproteins containing 1 ng of either IR or IGF-I-R were preincubated with or without increasing concentrations (1–100 nM) of either insulin or IGF-I for 1 h at 20 C in the presence of 2 mM $MnCl_2$, 10 mM $MgCl_2$, [$\gamma$-$^{32}$P]ATP (37 kilobecquerels/tube). The reaction was started by the addition of PGT (2.0 mg/ml). After 1 h at 22 C, 10 $\mu$l of the reaction mixture were spotted on filter paper ($2 \times 2$ cm; Whatman 3MM, Clifton, NJ), immersed in 10% trichloroacetic acid solution at 4 C containing 10 mM pyrophosphate. After extensive washing, the mixture was dried, and the radioactivity was counted with a liquid scintillation counter. Radioactivity in the absence of PGT was considered $^{32}$P nonspecific incorporation and was less than 15% of the basal activity detected in the presence of the peptide (18).

### *Measurement of PC-1 activity*

PC-1 activity was evaluated by measuring the hydrolysis of phosphosulfate bonds using the synthetic substrate 3′-phosphoadenosine, 5′-phosphosulfate (PAPS), as previously described (25). Briefly, 80% confluent cell monolayers incubated in the absence or presence of various doses of IL-1s for 6, 24, or 48 h were harvested and solubilized in 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 20 mM imidazole (pH

7.8) for 1 h at 4 C. Supernatants containing 0.1–2 μg protein were then incubated with 9 nmol [$^{35}$S]PAPS in the presence of 0.1 μmol $MgCl_2$ in 20 μl buffer (0.1 M 2-amino-2-methyl-1-propanol-HCl, pH 9.4) at 37 C. After 30 min, 25 μl 0.1 M sodium acetate (pH 5.5) were added, and samples were boiled for 1 min. Then, 0.5 ml activated charcoal was added, tubes were centrifuged, and supernatants were counted.

### *Statistical analysis*

Growth differences between control and IL-1-treated groups were analyzed for statistical significance by one-way ANOVA with Scheffe's F test for multiple comparisons using the statistical program StatView 512$^+$ (Abacus Concepts, Berkeley, CA). A two-way ANOVA was used to compare IR or IGF-I-R kinase activity in control and IL-1-treated groups.

## Results

### *Effects of IL-1α and IL-1β on MCF-7 cell growth stimulated by insulin or IGF-I in monolayer cultures*

MCF-7 cells have functional IR and IGF-I-R (14, 15), and both insulin and IGF-I have a mitogenic effect on these cells (16, 17). In concert with these previous observations, we found that both insulin and IGF-I increased cell growth in MCF-7 cell monolayers cultured in serum-free, estrogen-depleted medium. The effects of both insulin and IGF-I were dose dependent and maximal at 100 nM. In time-course studies, a significant effect was seen on day 3, and a maximum effect was seen on days 5–7 for both insulin and IGF-I (+53 ± 3% and +75 ± 5%, respectively).

When cells were grown in the presence of either IL-1α or IL-1β (100 U/ml), measurement of DNA cell content indicated that both IL-1s markedly antagonized the mitogenic effect of 100 nM insulin or IGF-I at all time points (not shown). The effects of the two IL-1s were similar, with IL-1β being slightly more potent than IL-1α. Therefore, IL-1β was used for all subsequent experiments. When MCF-7 cell monolayers were stimulated by either insulin or IGF-I (100 nM) and incubated with increasing concentrations of IL-1β (0–500 U/ml), cell DNA measurement on day 5 indicated that IL-1β significantly antagonized the mitogenic effect of both insulin ($P = 0.0052$; Fig. 1A) and IGF-I ($P = 0.0034$; Fig. 1A) in a dose-dependent manner. Inhibition was already observed at a dose of 50 U/ml ($P < 0.05$), and the maximal effect was observed at 500 U/ml. With the highest IL-1s concentrations, a slight, but not statistically significant, inhibition of MCF-7 cell proliferation was also observed in the absence of growth factor stimulation. No evidence of cytotoxicity was observed in cells incubated with IL-1s. Similar results were obtained in parallel experiments carried out in cells cultured in medium supplemented with 3% charcoal-stripped FCS.

### *Effects of IL-1α and IL-1β on MCF-7 anchorage-independent cell growth stimulated by insulin or IGF-I*

Colony formation was assessed 10 days after seeding cells in 0.33% agar in the absence or presence of increasing doses (1–100 nM) of either insulin or IGF-I. Both insulin and IGF-I enhanced colony size and number in a dose-dependent manner, with a maximal effect at 10–100 nM (not shown). A concentration of 100 nM insulin or IGF-I was then used to study the effects of IL-1s on growth factor-stimulated colony formation. IL-1s significantly inhibited colony formation

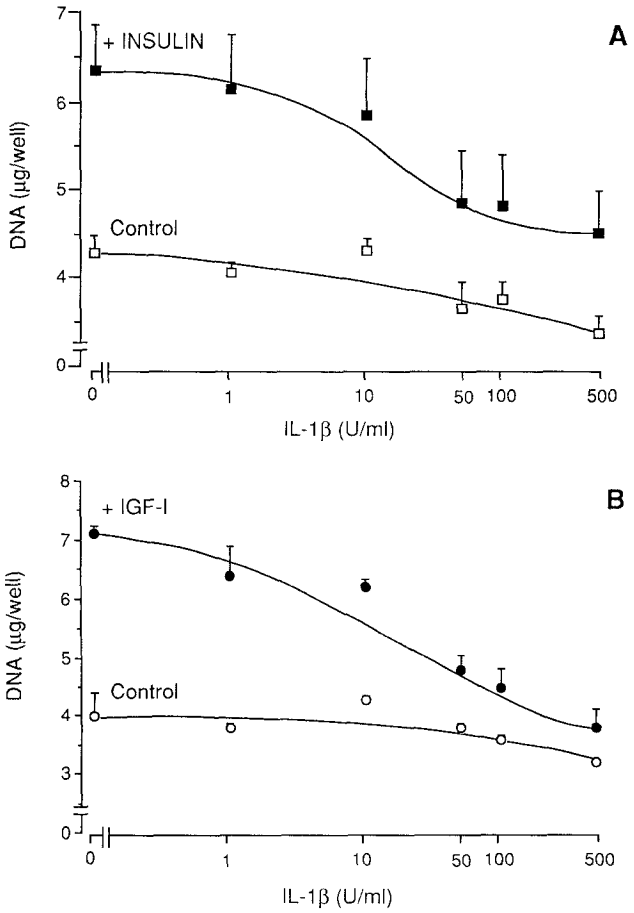


FIG. 1. IL-1β inhibition of MCF-7 cell growth stimulated by insulin or IGF-I. Cells were cultured in monolayers in serum-free estrogen-depleted medium, and cell DNA was measured after 5 days of incubation with IL-1β. Cell growth was stimulated with either 100 nM insulin (A) or 100 nM IGF-I (B). An IL-1β dose-dependent inhibition of growth was observed. Each *point* is the mean ± SEM of three separate experiments performed in triplicate.

stimulated by both insulin and IGF-I ($P = 0.0001$) in a dose-dependent manner. With IL-1β, an inhibitory effect was observed at 50 U/ml ($P = 0.002$), and the maximal effect was seen at 100–500 U/ml. Unstimulated colony growth was slightly decreased by exposure to IL-1β (Fig. 2, A and B).

### *Effect of IL-1β on IR and IGF-I-R protein and mRNA expression*

To evaluate whether IL-1β antagonized the insulin and IGF-I mitogenic effect by receptor down-regulation, we next measured IR and IGF-I-R content and binding characteristics in MCF-7 cells before and after exposure to IL-1β. First, both IR and IGF-I-R were measured by specific RIAs in solubilized MCF-7 cells after 24-, 48-, or 72-h exposure to 100 U/ml IL-1β. A significant increase of IR content ($P = 0.0057$) was observed in MCF-7 cells preincubated with IL-1β. The maximum effect was observed at 48 and with 100–500 U/ml. Under these conditions, IR content doubled compared with that in cells incubated without IL-1β (Table 1). In contrast, no significant change in IGF-I-R content was observed in MCF-7 cells incubated with IL-1β (Table 1).

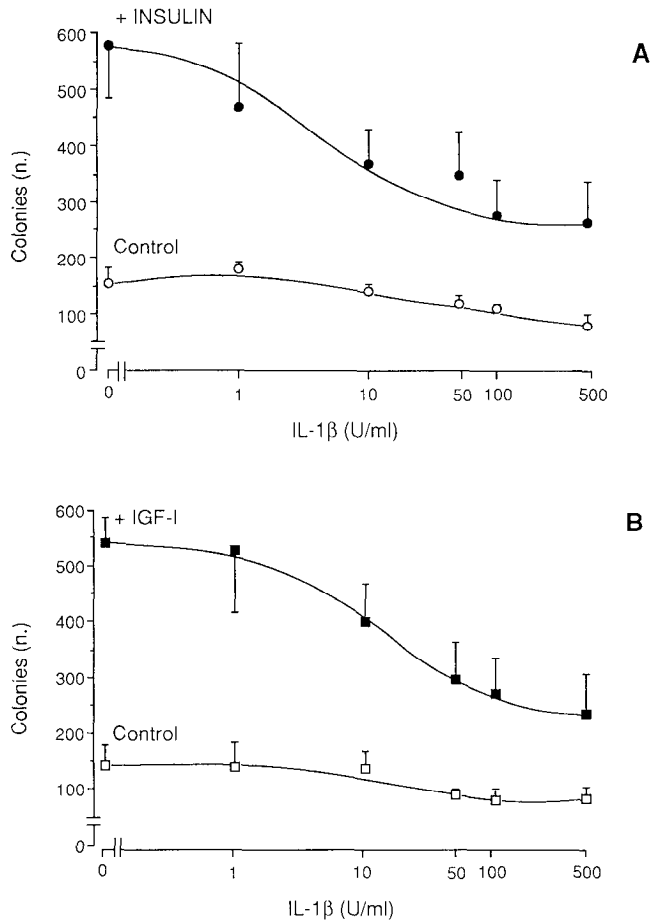


FIG. 2. IL-1β inhibition of MCF-7 cell anchorage-independent growth stimulated by insulin or IGF-I. Cells were seeded in 0.33% agar at $2 \times 10^4$ cells/35-mm dish in estrogen-depleted medium, and colony number (colony size, >30 cells) was scored after 10 days. Cells were stimulated with either 100 nM insulin (A) or 100 nM IGF-I (B). Each *point* is the mean ± SEM of three separate experiments performed in triplicate.

**TABLE 1.** Insulin and IGF-I receptor content determined by RIA in MCF-7 cells incubated with or without IL-1β for 48 h

| IL-1β (U/ml) | Insulin receptors (ng/$10^6$ cells) | IGF-I receptors (ng/$10^6$ cells) |
|---|---|---|
| 0 | 40.3 ± 5.5 | 274.7 ± 13.4 |
| 1 | 46.3 ± 12.9 | 268.0 ± 28.3 |
| 10 | 61.7 ± 12.2 | 286.0 ± 47.5 |
| 50 | 74.7 ± 14.3 | 295.7 ± 52.0 |
| 100 | 81.7 ± 8.5 | 295.3 ± 39.9 |
| 500 | 77.2 ± 16.6 | 266.7 ± 25.2 |

Data are expressed as the mean ± SD of three separate experiments 1 ng = $1.72 \times 10^9$ receptors (IR or IGF-I-R).

To assess whether the increase in IR protein induced by IL-1β was associated with an increased expression of the IR transcript, we measured IR mRNA. Poly(A)$^+$ RNA was extracted from MCF-7 cells cultured for 24 and 48 h with or without IL-1β (100 U/ml) and analyzed by slot blot. Densitometric reading of autoradiography indicated that the IR mRNA content was increased by 250 ± 12% (mean ± SD of two separate experiments) after 24-h incubation with IL-1β.

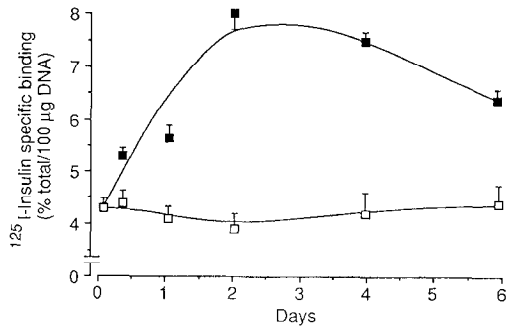


FIG. 3. Time course of the effect of IL-1β on [$^{125}$I]insulin binding to MCF-7 cells. Cells cultured in monolayers were first incubated with or without 100 U/ml IL-1β for the indicated times, and [$^{125}$I]insulin binding was then measured. A representative of three separate experiments, each performed in triplicate wells, is shown.

No further increase was observed after 48-h incubation. The level of the control gene (36 B4) was unchanged. In contrast to that of IR, the level of IGF-I-R mRNA did not change significantly after exposure to IL-1β.

*Effect of IL-1β on the function of the IR and IGF-I-R in MCF-7 cells*

As IL-1β antagonized insulin and IGF-I mitogenic effects in MCF-7 cells without decreasing the cell receptor number, we examined whether IR and IGF-I-R maintained their functional capacities after cell exposure to IL-1β.

*Binding studies.* We first ascertained whether cells incubated with IL-1β were able to bind insulin or IGF-I as well as control cells. MCF-7 cells were cultured in monolayers, and specific insulin or IGF-I binding was measured in cells preincubated in the absence or presence of 100 U/ml IL-1β for 6, 12, 24, 48, and 72 h. In concert with receptor content measurement by RIA, an increase in the specific insulin binding capacity was observed in MCF-7 cells exposed to IL-1β. The binding increase was already evident after 6-h exposure to IL-1β and was maximal after 48 h (Fig. 3). When cells were incubated for 48 h with increasing concentrations of IL-1β, a dose-response effect was observed, with a maximum increase of 200 ± 12% at 100 U/ml IL-1β.

Scatchard analysis of [$^{125}$I]insulin binding in MCF-7 cells indicated that IL-1β increased high affinity binding site capacity from 160 ± 60 to 450 ± 90 fmol/$10^6$ cells without any significant change in affinity ($K_d = 8.7 \times 10^{-9}$ *vs.* $9.4 \times 10^{-9}$). No significant variation in the low affinity binding sites was observed.

A similar increase in insulin binding was observed in cells incubated with insulin (100 nM) plus IL-1β (100 U/ml) compared with that in cells incubated with insulin alone. Specific IGF-I binding was not significantly changed in cells incubated with IL-1β.

*Receptor autophosphorylation and phosphotransferase activity.* We then examined the tyrosine kinase activity of IR and IGF-I-R, the first step of the intracellular signaling cascade of the two growth factors. Autophosphorylation of IR and IGF-I-R was measured in intact cells. Cell monolayers, preincubated or not with IL-1β (100 U/ml) for 48 h, were exposed to increasing concentrations of insulin or IGF-I for 10 min,

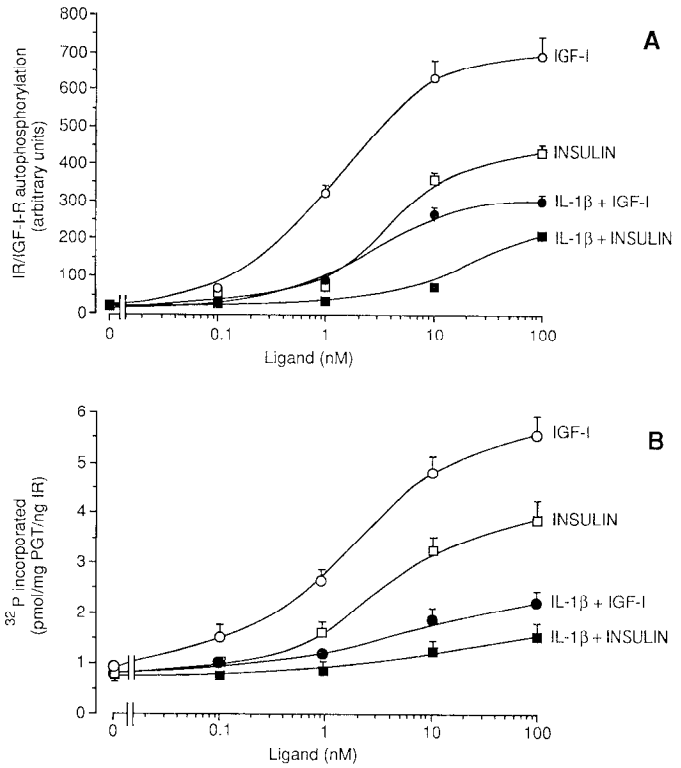


FIG. 4. A, Effect of IL-1β on ligand-stimulated autophosphorylation of the insulin or IGF-I receptor β-subunits in MCF-7 cells. Cell monolayers cultured in the presence or absence of IL-1β for 48 h were exposed to 100 nM of either insulin or IGF-I for 10 min. Cells were then lysed with buffer containing 1% Triton-X 100. Either IR or IGF-I-R were captured to plastic wells by specific antibodies. Phosphorylated receptors were revealed with a biotinylated phosphotyrosine antibody, followed by peroxidase-conjugated streptavidin. B, Effect of IL-1β on ligand-stimulated phosphotransferase activity of IR or IGF-I-R β-subunits in MCF-7 cells. WGA chromatography-purified glycoproteins were prepared from MCF-7 cell monolayers incubated with or without 100 nM IL-1β for 48 h. Insulin or IGF-I was then added to purified receptors (1 ng/tube) in the presence of [$^{32}$P]ATP and, $^{32}$P incorporation into PGT was measured as described in *Materials and Methods*. Each *point* is the mean ± SD of two separate experiments performed in triplicate.

then solubilized, and autophosphorylation of immunopurified receptors was measured by a sensitive plate capture assay. As shown in Fig. 4A, exposure to IL-1β markedly blunted autophosphorylation of both IR and IGF-I-R ($P$ = 0.0034 and $P$ = 0.0012, respectively) in MCF-7 cells. Similar results were obtained when IL-1α was used (not shown).

The phosphotransferase activity of IR and IGF-I-R was evaluated by measuring $^{32}$P incorporation into the synthetic substrate PGT. Cells incubated in the presence or absence of IL-1β for 48 h were solubilized, and semipurified receptors were prepared by WGA affinity chromatography. Solubilized receptors were then stimulated with either insulin or IGF-I in the presence of both labeled ATP and PGT, and $^{32}$P incorporated into PGT was measured. As shown in Fig. 4B, exposure to IL-1β almost abolished ($P$ = 0.0002) the phosphotransferase activity of both IR and IGF-I-R.

In contrast to the effect of IL-1β on receptor activity in intact cells (*in vivo*), the *in vitro* addition of IL-1β (50 U/ml) to isolated IRs from solubilized MCF-7 cells did not affect autophosphorylation or phosphotransferase activity.

*Studies on possible mechanisms of IL-1β inhibition of IR activity*

Many second messengers may be involved in mediating the effects of IL-1s, including TGFβ and $PGE_2$. To test whether TGFβ, which has been reported to be increased in MCF-7 cells incubated with IL-1 (11), may, in turn, impair the effect of insulin, intact MCF-7 cells were incubated with TGFβ at various concentrations (0.3–1 ng/ml) for 6, 12, or 48 h, and IR autophosphorylation was measured. TGFβ, however, did not affect insulin-stimulated IR autophosphorylation (data not shown).

We also evaluated whether the inhibitory effect of IL-1β was mediated by increased $PGE_2$ production. We measured insulin-stimulated cell growth and IR autophosphorylation in MCF-7 cells preincubated with IL-1β in the presence or absence of indomethacin. No significant effect on IL-1β inhibition of insulin action was caused by indomethacin (0.5–10 μM; data not shown).

We also explored the possibility that IL-1β caused insulin resistance by up-regulating the activity of membrane glycoprotein PC-1, also indicated as nucleotide pyrophosphatase/alkaline phosphodiesterase I, a multienzymatic membrane protein that has recently been shown to specifically inhibit the insulin-stimulated growth and IR tyrosine kinase activity (26). MCF-7 cells were incubated with or without IL-1β (50–100 U/ml), and PC-1 activity was measured in cell extracts after 6, 24, and 48 h. In cells incubated without IL-1β, the activity of this enzyme was 21 ± 3.5 nmol PAPS hydrolyzed/min·mg cell protein. These values were not significantly changed (24 ± 3.9) after incubation with IL-1β.

*Studies in nonepithelial cells overexpressing IRs*

We then evaluated whether IL-1β antagonizes the mitogenic effect of insulin in a different, nonepithelial, cell model. We used NIH-3T3 fibroblasts transfected with the IR gene and expressing high levels of IRs (3T3/HIR). In these cells, insulin promotes growth and induces colony formation in soft agar (20).

When 3T3/HIR cells were cultured in monolayers in the absence of serum and growth factors, IL-1β at a low dose (1–10 U/ml) had a proliferative effect (+35-40%; Fig. 5A). However, IL-1β markedly blunted the 3T3/HIR cell proliferative response to insulin, with a maximal effect at 500 U/ml (40–50% decrease after 48-h exposure; $P$ = 0.0003; Fig. 6A). When 3T3/HIR cells were seeded in soft agar, no colony formation occurred in the absence of insulin. Colony formation was induced by insulin in a dose-dependent manner, with a maximum effect at a dose of 100 nM. IL-1β reduced both the number and size of insulin-induced colonies in a dose-dependent manner ($P$ = 0.0004), with a maximal effect at 100–500 U/ml (−47%; Fig. 5B).

We then evaluated the effect of IL-1β on insulin-stimulated tyrosine kinase activity in 3T3/HIR cells. Cells cultured in monolayers in serum-free medium with or without 100 U/ml IL-1β for 48 h were evaluated for both IR autophosphorylation and phosphotransferase activity, as described for

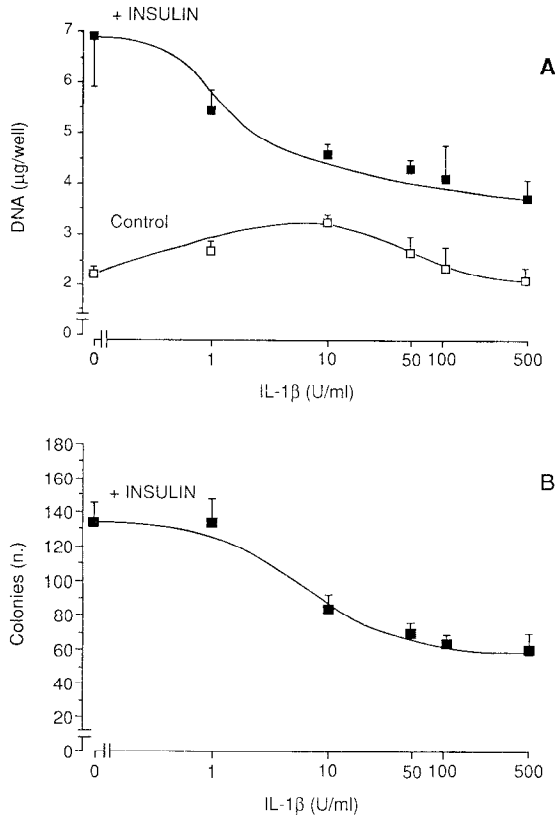


FIG. 5. Effect of IL-1β on insulin-stimulated anchorage-dependent and independent growth in 3T3/HIR cells. A, Cells were cultured in monolayers in serum-free medium with or without insulin, and cell DNA was measured after 5-day exposure to increasing concentrations of IL-1β. B, Cells were seeded in 0.33% agar at $2 \times 10^4$ cells/35-mm dish in the presence of 100 nM insulin and increasing concentrations of IL-1β. Colony number (size, >30 cells) was scored after 10 days. Each *point* is the mean ± SEM of three separate experiments performed in triplicate.

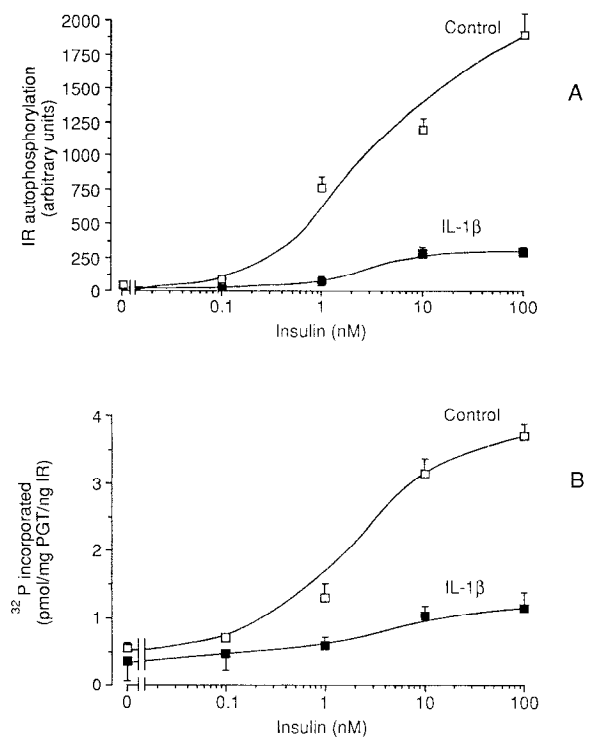


FIG. 6. Effect of IL-1β on insulin-stimulated autophosphorylation (A) and phosphotransferase activity (B) of IRs from 3T3/HIR cells. A, For autophosphorylation studies, cell monolayers cultured in the presence or absence of IL-1β for 48 h were exposed to 100 nM insulin for 10 min. Cells were then lysed, and IRs were captured to plastic wells by a specific antibody. Phosphorylated receptors were revealed with a biotinylated phosphotyrosine antibody followed by peroxidase-conjugated streptavidin. B, For phosphotransferase studies, WGA chromatography-purified glycoproteins were prepared from 3T3/HIR cell monolayers incubated with or without 100 nM IL-1β for 48 h. Insulin was then added to purified receptors (1 ng/tube) in the presence of [$^{32}$P]ATP, and $^{32}$P incorporation into PGT was measured as described in *Materials and Methods*. Each *point* is the mean ± SD of two separate experiments performed in triplicate.

MCF-7 cells. Similarly to what was observed in MCF-7 cells, both insulin-stimulated IR autophosphorylation and phosphotransferase activity were markedly reduced by IL-1β pretreatment ($P < 0.0001$; Fig. 6, A and B).

## Discussion

In the present study we found that IL-1s inhibit the mitogenic effect of both insulin and IGF-I in MCF-7 cells in a dose-dependent manner. This antimitogenic effect was observed both when cells were grown in monolayer cultures maintained in serum-free and estrogen-depleted medium and also when cells were seeded in soft agar, and insulin or IGF-I-stimulated colony formation was measured.

Previous studies have shown that MCF-7 cells possess specific binding sites for IL-1s. In these cells IL-1α induces growth inhibition and cell cycle arrest in the Go/G1 phase (10, 27). Although IL-1α down-regulates the estrogen receptor content in MCF-7 cells, the growth inhibition involves nonestrogen-regulated pathways, as IL-1α did not block estrogen-stimulated progesterone receptor content (13). The present findings suggest that the reported growth-inhibiting effect of IL-1s in MCF-7 breast cancer cells is due at least in part to inhibition of the growth-stimulating effect of insulin and/or IGF-I usually present in the culture medium.

The second main finding of this work is the inhibiting effect of IL-1s on IR and IGF-IR autophosphorylation. Both IR and IGF-IR are members of the tyrosine kinase growth factor receptor family (28), and autophosphorylation is the first step of the receptor signaling cascade (29). As a consequence of autophosphorylation inhibition, the phosphotransferase activity of IR and IGF-IR solubilized from IL-1-treated MCF-7 cells was inhibited.

In addition to the effect on receptor kinase activity, IL-1β up-regulated IR protein expression (but not IGF-IR expression) in MCF-7 cells. This IR up-regulation was associated with an increase in the specific mRNA, suggesting that either transcriptional control or altered mRNA stability is involved in this event. IR up-regulation may explain the less marked growth-inhibiting effect of IL-1 in insulin-treated cells than in IGF-I-treated cells (Fig. 1). The regulation of IR expression is a complex and not completely understood issue. IR up-regulation is induced by several events, including growth inhibition (30), cell differentiation (31), reduction of cellular ATP levels (32), and glucocorticoid treatment (33, 34). Similar

to what was observed with IL-1, chronic treatment with dexamethasone induces IR up-regulation and decreases insulin-induced IR autophosphorylation in different model systems with a mechanism that is still unclear (33, 34).

The inhibitory effect of IL-1$\beta$ on insulin-stimulated growth and IR tyrosine kinase activity was not restricted to epithelial MCF-7 breast cancer cells. In NIH/3T3 cells transfected with the IR cDNA, insulin stimulates the proliferation of monolayer cultures and also induces a ligand-dependent transformed phenotype, as shown by colony formation in soft agar (20). In these cells IL-1$\beta$ slightly stimulated cell growth in the absence of insulin, but clearly inhibited insulin-stimulated growth and colony formation. Again, also in this nonepithelial cell type, insulin-stimulated IR autophosphorylation and phosphotransferase activities were impaired.

IL-1s had no effect on the kinase activity when added *in vitro* to solubilized IRs. We, therefore, investigated some possible mediators of ILs-induced insulin resistance. IL-1s, in fact, are multifunctional cytokines that activate multiple second messengers (2). We found that stimulation of $PGE_2$ or TGF$\beta$ synthesis was not involved in the IL-1-induced insulin resistance. Furthermore, no up-regulation of PC-1 glycoprotein (an endogenous inhibitor of IR tyrosine kinase activity) (26) was observed after cell exposure to IL-1$\beta$. Induction of TNF$\alpha$ synthesis is also an unlikely mechanism. In fact, although IL-1 treatment in MCF-7 has been reported to induce TNF$\alpha$ mRNA, this induction is transient and is not accompanied by secretion of detectable TNF$\alpha$ protein (12). Other possible mechanisms by which IL-1 could inhibit IR and IGF-I-R tyrosine kinase include induction of free oxygen radicals and/or ion fluxes, cAMP increase, nitric oxide formation, or altered gene transcription via the DNA-binding protein nuclear factor-$\kappa$B (2, 3).

The potential therapeutical relevance of these effects of IL-1s in breast cancer is suggested by evidence that insulin- and IGF-I-stimulated pathways are important in the biology of breast cancer, as 1) both IRs and IGF-I-Rs are expressed at high levels in human breast carcinomas (35, 36); 2) overexpression of both IRs and IGF-I-Rs induces a transformed cell phenotype in NIH-3T3 and other cell types (20, 37); 3) peptide analogs of insulin may induce breast cancer in rats and premalignant changes in cultured human breast cells (Ref. 38 and our unpublished data); 4) IGF-I has been shown to be produced locally by stromal cells in human breast cancer and, therefore, to act as a paracrine factor (39); and 5) both insulin and IGF-I are potent mitogens in most human breast cancer cells and synergize the growth effect of estrogens (14, 40).

Combinations of cytotoxic chemotherapeutic agents and biological response modifiers are currently being explored for the treatment of cancer (41, 42). The present finding of the inhibiting activity of IL-1s on IR and IGF-I-R function may be of relevance in designing therapeutic strategies in breast carcinomas overexpressing IRs and/or IGF-I-Rs.

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