Progestins Increase Insulin Receptor Content and Insulin Stimulation of Growth in Human Breast Carcinoma Cells

Vincenzo Papa, Constance C. Reese, Antonio Brunetti, Riccardo Vigneri, Peniti K. Sitteri, and Ira D. Goldfine

Division of Diabetes and Endocrine Research, Mount Zion Medical Center of the University of California, San Francisco, California 94120; V. P., A. B., I. D. G.; Department of Obstetrics, Gynecology, and Reproductive Sciences (C. C. R., P. K. S.); and Department of Medicine and Physiology (I. D. G.), University of California, San Francisco, California 94143; and Istituto di Endocrinologia dell'Università di Catania, Ospedale Garibaldi, 95123 Catania, Italy (R. F.)

ABSTRACT

The effects of progesterone on the growth of breast carcinoma cells are undefined. In the present study we investigated the effect of progesterone on insulin receptor gene expression and insulin action in human breast cancer cells. Treatment of T47D cells with the synthetic progestin R5020 induced a time- and dose-dependent increase in insulin receptor content as measured by both ligand-binding studies and radioimmunoassay. Binding was half-maximally stimulated at 300 pm R5020 and maximal levels were reached after 4 days of treatment. Progesterone was 10-fold less potent than R5020. Cortisol had no effect on insulin receptor levels, while 17β-estradiol and dihydrotestosterone had minimal effects. Progestin treatment both increased insulin receptor mRNA levels and altered the relative distribution of the multiple insulin receptor mRNA transcripts. In order to study the functional significance of the increased insulin receptor levels, we incubated T47D cells with progesterone and then treated them with insulin. Insulin alone had a small effect on cell growth; however, the effect of insulin was markedly potentiated by progesterone treatment. These studies in breast cancer cells demonstrate, therefore, that insulin receptor gene expression is under the regulation of progestins and raise the possibility that progestin-insulin interactions may regulate breast cancer cell growth in vivo.

INTRODUCTION

The effects of progesterone on the growth of cancer cells are undefined. Both in vivo and in vitro studies have indicated that progesterone antagonizes estrogen-stimulated growth of endometrial cancer cells and normal uterine epithelial cells. This effect occurs by down-regulation of estrogen receptors (1–3). However, when similar studies were carried out with breast cancer cells, progesterone or synthetic progestins either inhibited (4, 5), stimulated (6), or had no effect (7) on cell growth. One explanation for these conflicting results in breast cancer cells is that there are interactions between progesterone and growth-promoting hormones which have not been elucidated.

Insulin is a known growth-promoting hormone for breast tissues. In cell culture insulin stimulates the growth of both human breast cancer cells (8) and normal human breast epithelial cells (9). However, whether insulin and progesterone interact to regulate breast cancer cell growth has not been explored.

In the present study, we have investigated the effect of progesterone on insulin receptors in T47D human breast cancer cells. Herein, we demonstrate that progestin treatment of these cells increases insulin receptor gene expression resulting in a twofold increase in insulin receptor content. Moreover, we find that progestin treatment of T47D cells enhances their growth response to insulin. These results suggest, therefore, that progesterone may stimulate the growth of breast cancer cells indirectly by increasing their sensitivity to insulin.

MATERIALS AND METHODS

Materials. The following chemicals were purchased: Hepes, bacitracin, phenylmethylsulfonyl fluoride, hydroxymethyl-methylglycine (tricine), and all steroids from Sigma Chemical Co. (St. Louis, MO); bovine serum albumin (fraction V) from Behring (Chicago, IL); porcine insulin from Eli Lilly and Co. (Indianapolis, IN); [125I]labeled insulin (2200 Ci/mmol), [32P]ATP (6000 Ci/mmol) and [32P]CTP (3000 Ci/mmol) from New England Nuclear (Boston, MA); oligo(dT) (12–18) from Pharmacia Laboratory Division (Uppsala, Sweden). All other reagents were of analytical grade. MA 10, a monoclonal antibody to the human insulin receptor, was prepared as previously described (10). IGF-1 was a gift from Ciba-Geigy (Summit, NJ). Insulin receptor cDNA probes were a gift of Dr. G. I. Bell (University of Chicago); A-lamin cDNA probe was a gift of Dr. W. Miller (University of California, San Francisco).

Cell Culture. T47D cells (from Dr. C. Sonnenschein and Dr. A. Soto, Tufis University, Boston, MA) and MCF-7 cells (from Dr. W. Nelson-Rees, Oakland Naval Biosciences Center, Oakland, CA) were routinely grown in DMEM containing 5% fetal calf serum, nonessential amino acids, glutamine, transferrin, penicillin, streptomycin, and biotin (Cell Culture Facility, University of California, San Francisco).

Growth Studies. For growth experiments 2 × 10⁶ cells/well were plated in 6-well plates in phenol-red-free DMEM supplemented with 2% human serum stripped twice with 2.5% (v/v) dextran-coated charcoal at 37°C for 90 min. After 1 day of culture, cells were pretreated for 48 h with 30 nm progesterone. Fresh hormone was added every 24 h. Then, varying doses of insulin were added every 12 h for 6 days. In some experiments, progestosterone treatment was continued with insulin treatment throughout the entire culture period. Cells were then harvested and plasma membranes lysed with 2 drops of Counta-Count (Diagnostic Technology, Hauppauge, NY). Nuclei were fixed with Boin's fixative and counted in a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

125I-Insulin Binding. For binding experiments in intact cells, layers were harvested with 1 mM EDTA and then re-suspended at 2–3 × 10⁶ cells/ml in binding buffer (DMEM containing 1% bovine serum albumin-25 μM tricine-25 mM Hepes, pH 7.8). Binding studies were carried out in binding buffer containing 125I-insulin (12 pm) or with or without unbound insulin (100 pm–1 μM). After incubation for 3 h at 16°C the cells were washed and dissolved in 0.3 mg/ml sodium dodecyl sulfate, and the radioactivity counted.

When binding studies were performed on solubilized receptors, the cells were solubilized with 1% Triton X-100 in the presence of protease inhibitors and 125I-insulin binding was then carried out as previously described (11).

Nonspecific binding (<1% of added ligand) was subtracted from total binding and binding data were normalized for DNA (12) content.

Insulin ReceptorRIA. To measure the insulin receptor content by RIA, cells were solubilized in 50 mM Hepes, pH 7.4–1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride-10 μg/ml Bactoarmin for 60 min

Received 5/1/90; accepted 9/13/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the John A. Kerner Foundation: USPHS Grant (CA 35852 A) awarded by the National Cancer Institute, DHHS; and Associazione Italiana Ricerca sul Cancro.

2 To whom reprint requests should be addressed, at Division of Diabetology and Endocrine Research, Mount Zion Medical Center, P.O. Box 7921, San Francisco, CA 94120–7921.

7858
INSULIN RECEPTOR REGULATION BY PROGESTINS

st 4°C. The lysate was then centrifuged for 10 min at 11,000 × g and the supernatant was used for RIA without further purification as previously described (13).

Preparation of RNA. Total cellular RNA was extracted from 3T3-cells according to the guanidinium isothiocyanate/cesium chl-14cleavage method (14). Poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography (15).

Slot Blots and Northern Transfers. For Northern transfers, poly(A)* RNA was denatured in formaldehyde, subjected to electrophoresis in 1% agarose, and transferred to nitrocellulose paper (15). For slot blots poly(A)* RNA was denatured as described by Thomas (16), and dilutions of RNA were immobilized on nitrocellulose with a Schleicher and Schuell slot blot apparatus. These studies used two human insulin receptor cDNA probes, 18.2 and 13.2 (1 and 4.2 kilobases, respectively), a kind gift from Dr. G. I. Bell (University of Chicago). They span the entire open reading frame of the receptor and extend into the 3' untranslated region (17). These were labeled by nick-translation (15) to a specific activity of 10^6 cpm/µg. The nitrocellulose filters were precintilation, hybridized, and washed as previously described (15). A probe for the nuclear envelope protein A-lamin (18) was labeled and hybridized as described for the insulin receptor. Poly(A)* RNA content was normalized by using an oligo(dT) that was end labeled using the enzyme T4 polyribonucleotide kinase (19).

RESULTS

Studies in T47D Cells

Progestin Effects on 125-I-Insulin Binding

Insulin-binding Specificity. We first characterized the insulin receptors in T47D cells by using 125-I-insulin and unlabeled ligands in competition-inhibition studies (Fig. 1). T47D cells bound 125-I-insulin and binding was competed for by unlabeled insulin at an ED50 of 0.5 nm. The ED50 for the related hormone IGF-1 was >10 nm. Monoclonal antibody MA-10, which inhibits insulin binding to the insulin receptor but not IGF-1 binding to the IGF-1 receptor (10), had an ED50 of 2.0 nm. Normal mouse IgG had no effect. The maximal specific binding of 125-I-insulin to T47D cells was 1.12 ± 0.14% of mg protein or 2.51 ± 0.32%/100 µg DNA (mean ± SE, n = 10).

Time Course and Specificity of the Steroid Efect. Progesterone and its synthetic analog R5020 increased insulin binding to T47D cells in a time- and dose-dependent manner. 125-I-

[Figures and diagrams are not transcribed.]
of gene transcription (21,22), we examined whether R5020 treatment was associated with an increase in insulin receptor mRNA levels. Poly(A)⁺ RNA from control and R5020-treated cells was slot blotted, and the filters were probed with labeled insulin receptor cDNA (Fig. 4A). After a 4-day incubation with 100 nM R5020 there was an approximately 2-fold increase in insulin receptor mRNA content. In contrast, treatment with R5020 had no effect on mRNA levels of the control A-lamin, a protein whose cellular content is not readily altered by changes in growth conditions (18).

Insulin receptor mRNA consists of several species of different molecular sizes (23). In order to determine whether R5020 treatment was associated with a qualitative difference in insulin receptor mRNA, poly(A)⁺ RNA was subjected to agarose gel electrophoresis, followed by transfer to nitrocellulose filters. These filters were then probed with labeled insulin receptor cDNA. In T47D cells, 3 major bands of insulin receptor mRNA were seen at 11.0, 8.5, and 7.5 kilobases (Fig. 4B). After 4 days of treatment with 100 nM R5020 the relative amount of these 3 insulin receptor mRNA species was significantly changed (Fig. 4B). Quantitative scanning of bands revealed that in control cells the most prominent mRNA species were the 8.5- and 7.5-kilobase species; in contrast, after R5020 treatment the most prominent species were the 11.0- and 8.5-kilobase species.

Effects of Progestins on Insulin Stimulation of Cell Growth

In order to determine whether the progesterin-induced increase in insulin receptor content was associated with enhanced insulin action, we studied the growth of T47D cells (Fig. 5). Cells were continuously treated with 30 nM progesterone and then with or without insulin. In the absence of insulin, progesterone had a nonsignificant effect on growth. Insulin, in the absence of progesterone, increased growth by about 10%. In contrast, progesterone and insulin together had a synergistic effect, stimulating growth by 45%. In progesterone-treated cells, insulin had a detectable effect on growth at 100 pm and a maximal effect at 10 nm (Fig. 5).

Studies in MCF7 Cells

In order to establish that the progesterone effect on insulin action occurs in other breast cancer cell lines, we studied MCF7

### Table: Insulin Receptor mRNA and Lamin mRNA Levels

<table>
<thead>
<tr>
<th>Condition</th>
<th>Insulin Receptor mRNA (ng)</th>
<th>Lamin mRNA (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>R5020</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of R5020 on insulin receptor mRNA. T47D cells were grown for 2 days without R5020 and then incubated 4 days in the absence or presence of 100 nM R5020. A, slot blot of poly(A)⁺ RNA from control and R5020-treated cells. Filters were probed with labeled insulin receptor cDNA. B, Northern blot of insulin receptor poly(A)⁺ RNA from control (+) and R5020-treated (−) cells. A representative of 4 different experiments is shown. An RNA ladder was used as the molecular weight standard. KB: kilobase-

Progestin Effects on Insulin Receptor mRNA

Since progestins and other steroid hormones increase the rate binding. Since these plots were curvilinear, we analyzed binding by a two site model (20). Treatment for 4 days with 100 nM R5020 increased the total binding capacity of T47D cells (9750 ± 1520 versus 4240 ± 1120 sites/cell, n = 3) and the capacity and affinity (0.54 ± 0.03 versus 1.47 ± 0.10 nM, n = 3) of the high affinity binding sites.

As an alternative method for measuring the insulin-binding capacity we used an insulin receptor radiolmmunoassay as recently described (13). This assay is specific for the insulin receptor; insulin has no cross-reactivity. The related insulin-like growth factor I has <3% cross-reactivity (13). Assay of solubilized extracts from both control cells and cells treated for 4 days with 100 nM R5020 produced dilution slopes that were parallel to the purified human placenta standard (Fig. 3B). R5020-treated cells had a >2-fold higher concentration of insulin receptors measured by radiolmmunoassay than control cells (8.77 ± 1.02 versus 4.12 ± 0.48 ng of insulin receptor/10⁴ cells, respectively; n = 3).

Fig. 3. Effect of R5020 on insulin receptor content in T47D cells. Cells were grown for 2 days without R5020 and then incubated for 4 days in the absence or presence of 100 nm R5020. A, ¹²⁵I-insulin binding. A representative of 3 experiments is shown. Inset, Scatchard plot of the data; insulin bound = females/100 μg DNA. B, radioimmunoassay of insulin receptors. Competition-inhibition curves for extracts of control (○) and R5020-treated (△) T47D cells on the binding of ¹²⁵I-insulin receptors to an antireceptor antiserum. A representative of 2 experiments is shown.
INSULIN RECEPTOR REGULATION BY PROGESTINS

Fig. 5. Effect of progestosterone on the growth response of T47D cells to insulin. On the left, cells were continuously incubated after 1 day of culture in the presence or absence of 30 nM progestosterone for the entire experiment. After 2 days of treatment, 10 nM insulin was added for 6 days. P cells treated with progesterone; L cells treated with insulin; P + L cells treated with progesterone and insulin. On the right, cells were incubated for 48 h in either the presence (+P) or absence (−P) of 30 nM progesterone and then treated for 6 days with different doses of insulin. Cells were harvested and cell nuclei counted as described in “Materials and Methods.” Points and columns, means of 3 separate experiments in which at least 3 replicate wells were counted; bars, ±SE; control cell number, mean cell number/well on day 9 of culture with 0.001% ethanol vehicle only. The cell doubling time of such unstimulated control was approximately 70 h.

Fig. 6. Effect of progesterone on the growth response of MCF7 cells to insulin. MCF7 cells were preincubated in the presence (+P) or absence (−P) of 30 nM progesterone and then treated with insulin as described for T47D cells in Fig. 5. Points, means of 3 separate experiments performed with triplicate determinations; bars, ±SE; control cell number, mean cell number/well on day 9 of culture with 0.001% ethanol vehicle only.

cells, a cell line known to be regulated by insulin (24). Treatment with progesterone for 3 days increased insulin receptor content by 40–50% (data not shown). As in T47D cells, progesterone potentiated insulin stimulation of cell growth (Fig. 6).

DISCUSSION

In the present study we find that treatment of human breast carcinoma cells with progestins increases insulin receptor gene expression and insulin receptor content. As a consequence, insulin stimulation of cell growth is enhanced. T47D cells were chosen for the majority of these studies for two reasons. First, it was previously reported that progestin treatment increases insulin receptor binding in this cell line (5). Second, these cells express high basal levels of progesterone receptors (25) and, therefore, estrogen treatment is not needed for progesterone receptor gene expression as is the case with other normal and cancer cell lines.

The insulin receptor is an αβ2-heterotetramer with two extracellular α subunits (Mf, 130,000) and two transmembrane β subunits (Mf, 90,000) that contain tyrosine kinase activity in their intracellular domains (23). One α and one β subunit are derived from a common precursor that is proteolyzed into separate subunits in the Golgi (23). This insulin receptor precursor is derived from a large single gene of 130 kilobases on chromosome 11q23 (26). This gene has 22 exons.

Insulin receptor mRNA content in T47D cells was increased by progestin treatment. Moreover, the expression of insulin receptor mRNA was altered in a qualitative manner. In most cells that have been studied, several insulin receptor mRNA species are observed in agarose gels with molecular sizes ranging widely from 5.2 to 11 kilobases (23). Studies of these multiple transcripts suggest that they are due to variable splicing at the 3′ end of the insulin receptor RNA (23, 27). There are multiple 5′ start sites but they differ only by several hundred bases (28) and thus do not account for the size heterogeneity. While these multiple species are all involved in insulin receptor protein synthesis (since they are all found associated with ribosomes) (22), the biological role for these multiple mRNA species is unknown. In T47D cells, we observed that progestin treatment increased the relative content of the higher molecular weight mRNA forms. While progestins and other steroid hormones act primarily to increase gene transcription (21, 22), in some instances they have been reported to alter RNA processing and turnover (21, 29, 30). The present study of the insulin receptor in T47D cells provides a new example, therefore, of steroid hormone regulation of mRNA metabolism.

In the present study we observed that the increment in insulin receptor mRNA content was paralleled by an increase in insulin receptor protein content as measured by both Scatchard analysis and radioimmunoassay. In addition to changing binding capacity, progestin treatment also altered the affinity of the high affinity insulin-binding sites. The reason for this effect is unknown but may reflect an effect of progesterins on other elements of the plasma membrane such as lipids which are known to influence receptor affinity (23).

Progestins also increase insulin binding to its receptor in ZR-75 human breast cancer cells (31) and in a variant subline of T47D (5), but the mechanism of this up-regulation was not reported. Also, we have now observed that progesterone pre-treatment of both T47D cells and MCF7 cells enhances insulin stimulation of cell growth. This observation indicates that up-regulation of insulin receptors by progestin in human breast cancer cells is directly related to enhanced insulin action.

The present observations demonstrating an interaction between insulin receptor and steroid receptors in cultured human breast carcinoma cells may have clinical implications. We have recently observed that insulin receptors are elevated 7-fold in breast carcinomas when compared with normal breast tissues (32). It is possible, therefore, that the interaction between progesterone and insulin receptors in breast cancer cells may also occur in vivo and this interaction may have a role in regulating the growth of breast tumors (33). These findings may also be relevant to the proliferation rate of normal breast epithelial cells. In rodent progesterone, in the presence of insulin, stimulates the growth of cultured mammary epithelial cells (34). Also, several studies have shown that the mitotic activity of normal breast epithelial cells is highest during the luteal phase of the menstrual cycle when progesterone levels are highest (35).

REFERENCES

1. Lane, G., Siddie, N. C., Ryder, T. A., Pryso-Davies, J., King, R. J. B., and Whitehead, M. J. Effects of hydrocortisone on the osteogened postmeno-


