

Abnormal Insulin-Receptor Down Regulation and Dissociation of Down Regulation from Insulin Biological Action in Cultured Human Tumor Cells¹

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ABSTRACT

The sensitivity of insulin receptors to down regulation by insulin has been measured in cultured human tumor cells (breast tumor cell lines MCF-7, T-47D, and colon tumor cell line HCT-8). Insulin receptors on breast tumor cells were resistant to down regulation (15–17% maximum loss of insulin binding after 4 h exposure to 170 nM insulin). HCT-8 cells were sensitive to down regulation after 4 h exposure to 3.8 nM insulin, but the extent of down regulation then lessened at higher concentrations of insulin. This paradoxical behavior was associated with increasing affinity of insulin receptors for insulin following exposure to hormone. Insulin-stimulated [³H]leucine incorporation into protein was measured in parallel with studies of receptor regulation to assess the effect of preexposure of cells to insulin on cell metabolism. Maximum down regulation of receptors in all three types of tumor cell by prior exposure to insulin did not significantly alter the responsiveness of any of the cell lines to insulin. Thus insulin receptor down regulation is abnormal in these tumor lines compared with reported studies in normal cells, and this may contribute a metabolic advantage to these malignant cells over normal tissues.

INTRODUCTION

Insulin receptors have been detected in a variety of neoplastic and nonneoplastic cultured cells. In many instances these receptors have been shown to down regulate in response to increased ambient insulin concentrations (1–5). The sensitivity of receptors to down regulation appears to be a function of cell type, insulin concentration, and duration of exposure to hormone. It has been suggested that insulin receptors in tumor tissue may be less sensitive to down regulation *in vivo* than those in normal tissues (6). In addition, receptors in tumor tissue appear less sensitive to down regulation than those in normal cells when studied *in vitro* over a range of insulin concentrations for a fixed time period (7). These latter observations have been extended in this study by measuring down regulation as a function of duration of exposure to insulin as well as hormone concentration and assessing insulin receptor binding affinities and receptor number before and after down regulation. In addition, the biological response to insulin has been measured in parallel with binding studies. Down regulation of insulin receptors is thought to lead to decreased biological responsiveness to insulin (8–10), although there are also reports of increased insulin responsiveness as a consequence of hyperinsulinemia both *in vivo* and *in vitro* (11–13). In view of these conflicting reports the effect of insulin receptor down regulation on insulin-induced protein synthesis in cultured human tumor cell lines has been investigated to test whether preexposure to hormone desensitizes these cells to this biological action of insulin.

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MATERIALS AND METHODS

Highly purified porcine insulin was purchased from Novo Industri A/S (Copenhagen, Denmark). Carrier-free Na¹²⁵I and protosol were purchased from New England Nuclear (Boston, MA), bovine serum albumin (radioimmunoassay grade, Fraction V), and epidermal growth factor from Sigma Chemical Co. (St. Louis, MO), MEM⁴ (No. 410-2000) from Grand Island Biological Co., and fetal calf serum was purchased from Gibco NZ Ltd. L-[4,5-³H]Leucine (131 Ci/mmol) was obtained from Amersham International (Amersham, England).

Cell Culture. Tumor cell lines comprised two human breast carcinomas (MCF-7 and T-47D) and a human colon carcinoma (HCT-8). Cells were grown in plastic culture flasks (25 or 75 cm²; Falcon Plastics) on MEM supplemented with 10% FCS and equilibrated with 5% CO₂ in air. T-47D and HCT-8 cells were supplemented with 2 mM glutamine whereas MCF-7 cells required 6 mM glutamine. Porcine insulin (0.33 μM) and hydrocortisone (0.55 μM) were also added to MEM for culture of T-47D cells, while medium for growth of MCF-7 cells contained an insulin concentration of 1.6 μM unless stated otherwise. The tumor cell lines were passaged weekly with 0.1% trypsin. All cell lines were free of contamination by *Mycoplasma*.

To prepare cultures for binding studies, cells were seeded in 60- x 15-mm plastic culture dishes in 5 ml growth medium containing antibiotics and grown until almost confluent. MCF-7 and T-47D (2.5 x 10⁵ cells plated/dish) were used for binding studies on days 6 and 8, respectively, after seeding and HCT-8 (2.5 x 10⁴ cells plated/dish) were used on day 7.

Binding Assays. Insulin was iodinated to a specific activity of 54–124 μCi/μg using a modified chloramine-T method described previously (7). [¹²⁵I]insulin was separated from ¹²⁵I by gel filtration on a 1- x 60-cm Sephadex G 50 column equilibrated in 0.025 M Tris-HCl, pH 7.6. The peak fractions were pooled and kept at 4°C for a maximum of 5 days. All binding experiments were performed using monolayers grown in 60- x 15-mm plastic culture dishes. Because the presence of serum and hormones in the culture medium could potentially influence receptor numbers, cells were cultured in medium deprived of serum and hormones for 24 h prior to all binding experiments. Growth medium was aspirated, and the cells washed twice with 2 ml ice-cold binding buffer [100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]:120 mM NaCl:1.2 mM MgSO₄:2.5 mM KCl:15 mM NaC₂H₃O₂:10 mM glucose:1% bovine serum albumin, pH 7.8 at 22°C]. Monolayers were incubated in duplicate with [¹²⁵I]insulin (5.0 x 10⁻¹¹ M for equilibrium binding analysis, otherwise 1.8–2.0 x 10⁻¹⁰ M) in 2 ml binding buffer. Nonspecific binding was assessed in monolayers grown in parallel and incubated with [¹²⁵I]insulin and excess unlabeled insulin (2 μM). For equilibrium binding analysis, monolayers were incubated with a fixed concentration of [¹²⁵I]insulin and increasing concentrations of unlabeled hormone (0–1 μM). Cells were incubated at the temperatures and times indicated, after which they were washed three times with 2 ml ice-cold binding buffer. The cells were solubilized with 2 ml 1N NaOH at 37°C for 1 h. A 1-ml aliquot of the solubilized cells was counted in a Searle 1285 automatic gamma counter with 75% counting efficiency. Degradation of insulin under conditions used for down regulation was determined by spiking the culture medium with [¹²⁵I]-insulin and testing the ability of residual unbound [¹²⁵I]insulin to precipitate with an equal volume of 10% TCA.

Pretreatment of Cells with Exogenous Insulin before Receptor Binding Studies. In order to study insulin-induced receptor down regulation, unlabeled porcine insulin dissolved in MEM was added in 100-μl

⁴ The abbreviations used are: MEM, minimal essential medium (α modified); TCA, trichloroacetic acid; FCS, fetal calf serum.

aliquots to monolayers deprived of FCS and insulin for the previous 24 h, to give final concentrations of insulin ranging from 3.5×10^{-9} – 1.7×10^{-7} M. After incubation at 37°C for the times indicated, the medium was aspirated and the cells washed twice with 2 ml binding buffer. Surface bound insulin was dissociated from the monolayers to allow estimation of residual receptors by binding of [125 I]insulin.

Dissociation Procedure. Exogenous insulin bound to cell surfaces was allowed to dissociate into 2 ml of binding buffer at pH 7 for 1 h at 37°C. After incubation, cells were washed twice with 2 ml ice-cold binding buffer (pH 7.8) before estimation of binding of [125 I]insulin. This procedure removes >90% of surface bound insulin (7).

Pretreatment of Cells with Exogenous Insulin before Biological Assay. In order to study the effect of insulin-induced receptor down regulation on biological response to insulin, unlabeled porcine insulin dissolved in MEM was added in 100- μ l aliquots to monolayers deprived of FCS and insulin for the previous 24 h, to give final insulin concentrations of 1.7×10^{-8} (HCT-8) and 1.7×10^{-7} M (MCF-7 and T-47D). Control cells received 100 μ l MEM in place of unlabeled insulin. After incubation at 37°C for 4 h in order to achieve down regulation, the medium was aspirated and the cells washed twice with 5 ml MEM.

Biological Assay: Incorporation of [3 H]Leucine into Protein. [3 H]Leucine was added to monolayers in triplicate (0.25 μ Ci/ml) for 7 h at 37°C in the presence of 0–4.4 μ M insulin. Leucine incorporation was stopped by aspirating the medium and washing the cells twice with 2 ml cold phosphate buffered saline. Cells were harvested by incubation with 2 ml 0.05% trypsin for 5 min at 37°C together with mechanical scraping and transferred to glass test tubes. Protein was precipitated by adding 0.4 ml cold 50% TCA containing unlabeled leucine (50 mg/liter) to each tube on ice, and the precipitates were boiled for 10 min in order to hydrolyze acyl-tRNA. The precipitates were chilled on ice before being trapped on presoaked 2.5-cm glass microfiber filters (Whatman GF/B) and were then washed three times with 5 ml cold 5% TCA containing unlabeled leucine (5 mg/liter). The filters were dried in an oven at 90°C for 20 min. Precipitates were solubilized in 1 ml Protosol for 1 h at 55°C with shaking and after cooling, 100 μ l glacial acetic acid were added followed by 10 ml Triton:toluene (0.4:1) scintillation fluid, and radioactivity was measured in an LKB 1217 Rackbeta scintillation counter.

The parameters calculated from the dose response curves were the basal rate of [3 H]leucine incorporation into protein and the response of cells to insulin-stimulated protein synthesis. This was calculated at each dose of insulin according to the formula

$$\text{Response} = \frac{\text{Rate of incorporation} - \text{basal rate of incorporation}}{\text{Basal rate of incorporation}} \times \frac{100}{1}$$

The responsiveness of cells to insulin was compared between experiments by analysis of dose response curves as outlined under "Statistics" (see below).

Cell Counts. For each experiment, cells were grown in parallel to assay cultures in duplicate 60- x 15-mm plastic culture dishes and used exclusively to estimate cell counts. Cells were removed using 0.1% trypsin (37°C, 10 min). MCF-7 and T-47D cells were counted in a Model ZF Coulter Counter using a 100- μ m aperture. MCF-7 and T-47D cells were syringed several times before counting to provide single cell suspensions. HCT-8 cells were particularly susceptible to damage by syringing and thus were treated with 0.2% DNase (22°C, 10 min) to disperse clumps before counting in a hemocytometer.

Statistics. Student's *t* test for either paired or unpaired data, where appropriate, was used to determine significance of difference between two or more sets of specific binding data. Data from equilibrium binding experiments were analyzed on an IBM personal computer by the "Ligand" program (14). This method provided optimal weighted least squares estimates of binding parameters (high and low affinity constants K_a , high and low affinity binding capacities R , and nonspecific binding N). The program determined the goodness of fit to the observed data and calculated appropriate SEs for the parameters, thus providing an objective estimate of their precision.

Differences between the data obtained from the Ligand analyses of

pooled equilibrium binding experiments in Tables 1 and 2 were tested for significance by Student's *t* test and where appropriate by the Tukey Kramer multiple comparison test (15). The SEs determined by the Ligand program were used for comparing the parameters from pooled analyses. The variances used for comparing the parameters from individual Ligand analyses were derived from the SEs of the means of the parameters.

Differences between the means of basal rates of incorporation for different experimental conditions were tested for significance using the Tukey Studentized range test (16). Similarly, differences between the dose-response curves performed under different experimental conditions were tested for significance by taking the mean of the differences at each insulin concentration and using the Tukey Studentized range test. These analyses were performed on the University of Auckland mainframe IBM computer using the statistical analysis system package.

RESULTS

The rate of binding of insulin to receptor was similar for control cells and those with down regulated receptors (Fig. 1). The degradation of insulin by cells cultured for 7 h at 37°C in MEM ranged from 1–9%/10⁶ cells at physiological concentrations of insulin and from 0–1.1%/10⁶ cells at higher concentrations of insulin.

Down regulation of insulin receptors as a function of hormone concentration and time of exposure of cells to insulin is shown in Fig. 2. Receptors in both of the breast tumor cell lines were generally resistant to down regulation. Although significant loss of binding was observed at several time points at high insulin concentrations, at no time did binding decrease by more than 25%. By comparison, the human colon tumor cell line, HCT-8, appeared sensitive to down regulation with a significant binding loss of 46% after 4 h exposure to 3.8×10^{-9} M insulin. The sensitivity of insulin receptors to down regulation in HCT-8 cells was also time and dose dependent. Paradoxically, however, the degree of down regulation was inversely related to insulin concentration such that a maximum decrease of binding was observed after exposure to low concentrations (3.8×10^{-8} M) but almost no loss of binding was seen after exposure to high concentrations (1.7×10^{-7} M).

The breast tumor cell lines, T-47D and MCF-7 were routinely grown in the presence of 10% FCS, insulin, and hydrocortisone (T-47D only). The long-term exposure of these cells to elevated levels of insulin might have contributed to their resistance to down regulation despite removal of FCS and supplementary hormones 24 h prior to testing. Thus additional studies of insulin receptor down regulation were performed in these cells after growth in MEM and 5% FCS only. The cells grew to lower densities under these conditions than in the presence of supplemented medium. However, when insulin binding was measured as a function of hormone concentration and time of exposure to insulin a similar resistance to down regulation was seen to that observed under standard conditions (data not shown).

In order to assess whether down regulation of insulin receptors was related to receptor loss or a change in the affinity of insulin binding to receptor, equilibrium analysis of [125 I]insulin binding was performed before and after exposure of cells to insulin. Binding curves for cells not previously exposed to insulin are shown in Fig. 3. These have been generated from pooled data from 3–5 individual experiments using the Ligand program. Binding to T-47D cells showed best fit with a one-site binding model and MCF-7 and HCT-8 cells showed best fit with a two-site binding model (Table 1). The insulin binding affinity for T-47D cells was similar to that obtained for the low

Fig. 1. Time course of insulin binding. Cells were preexposed to 0 (●), 17 (○), and 170 (□) nM insulin for 4 h at 37°C; then exogenous insulin was dissociated at pH 7 for 1 h at 37°C before cells were incubated with [¹²⁵I]insulin with or without unlabeled insulin at 4°C for the times indicated on the abscissa. Data are means of duplicates shown for two separate experiments.

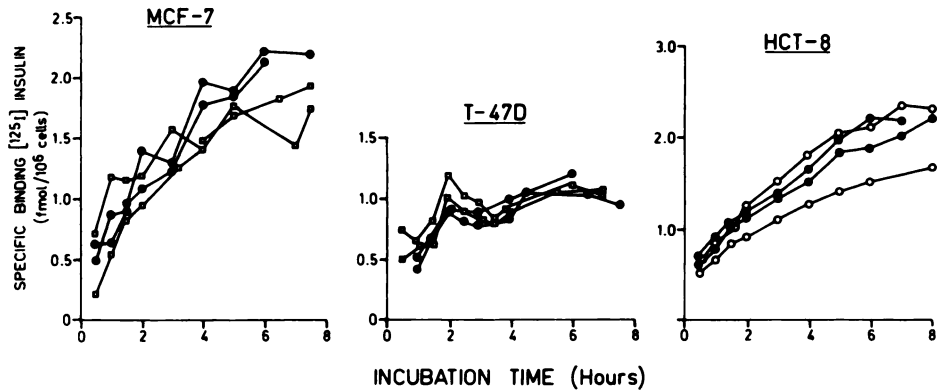


Fig. 2. Insulin receptor levels after preincubation of cells with various insulin concentrations for up to 7 h at 37°C (○, 3.8×10^{-9} M; +, 1.7×10^{-8} M; ×, 1.7×10^{-7} M insulin). After dissociation of exogenous bound insulin, cells were incubated with [¹²⁵I]insulin $\pm 2 \mu$ M unlabeled insulin for 4 h at 4°C. Data are expressed as a percentage change from control cells preincubated for the same period without insulin. Means \pm SE (bars) are shown; numbers in parentheses, number of experiments; *, $P < 0.05$ compared with control cultures.

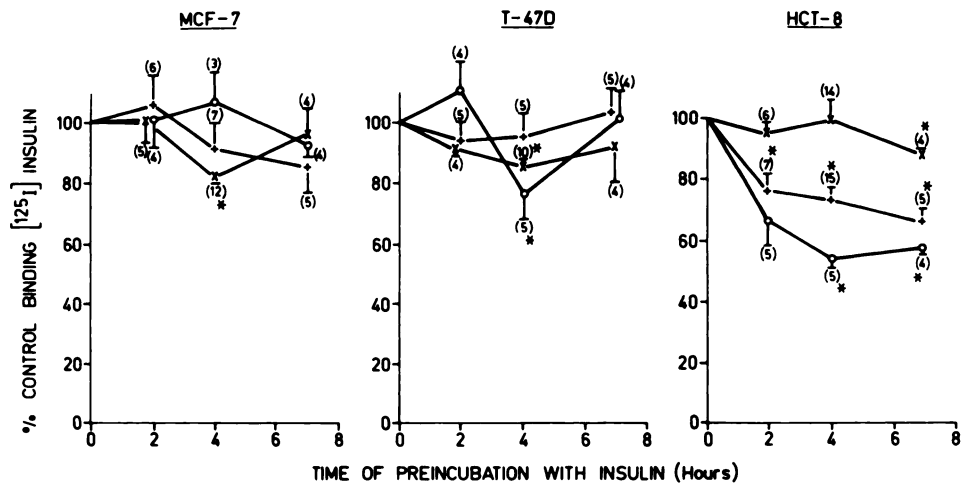
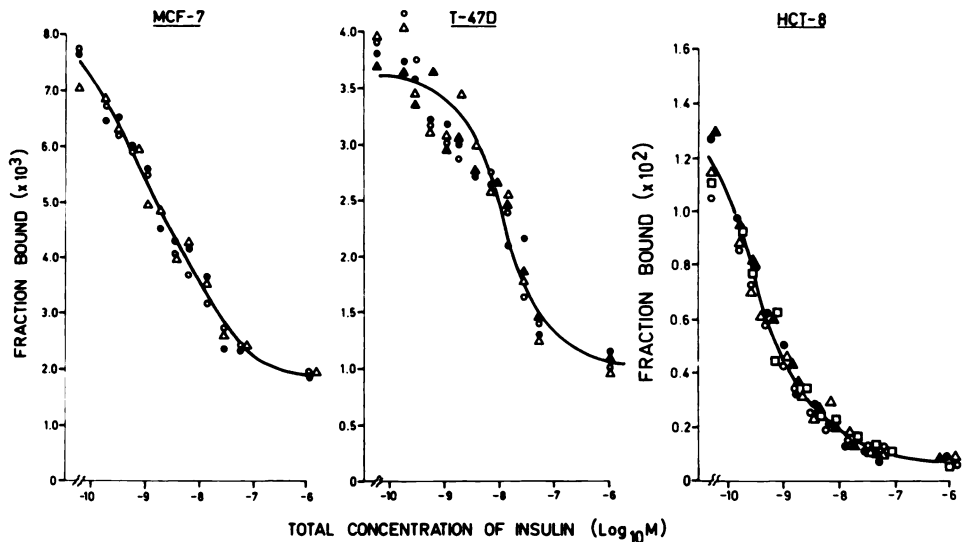


Fig. 3. Equilibrium binding of insulin to cells in basal state. Symbols, different experiments. Line of best fit computed by Ligand program.



affinity site of MCF-7 and HCT-8 cells. There was no significant change in binding parameters in the two breast tumor cell lines during attempted down regulation, as predicted from Fig. 2.

Down regulation in HCT-8 cells was associated with a trend for binding affinity to increase and receptor number to decrease, but in general these changes were not significant (Table 1). However, changes in both receptor number and binding affinity probably contribute to down regulation in this cell line, since a significant decrease in overall binding of insulin was observed during down regulation experiments (Fig. 2). Subsequently these data were also analyzed after fitting to a one-site binding model. Following preincubation with the lowest concentration of insulin, when the greatest reduction in overall binding of

insulin was observed (Fig. 2), receptor number was reduced with little change in binding affinity (Table 2). When the preincubation medium contained 1.7×10^{-8} M insulin the number of receptors decreased further, but there was an associated increase in binding affinity, which could account for the observed restoration of overall insulin binding as seen in Fig. 2. When the insulin concentration was raised further (1.7×10^{-7} M) there appeared to be both an increase in receptor number and a further increase in affinity, restoring overall insulin binding capacity to its control level. Insulin-induced down regulation of insulin receptors in these cells thus appeared complex and quite different from the pattern seen in normal cells (1, 2, 17).

These binding data were compared with the biological re-

Table 1 Equilibrium insulin binding before and after receptor down regulation derived from Ligand analyses of pooled data

| Cell | Insulin concentration used for down regulation (M) | No. of experiments | High affinity binding site | | Low affinity binding site | | Nonspecific binding (% bound) |
|-------|--|--------------------|---------------------------------------|---------------------------------|---------------------------------------|---------------------------------|-------------------------------|
| | | | $K_a \pm SE$ ($10^9 \times M^{-1}$) | $R \pm SE$ (fmol/ 10^6 cells) | $K_a \pm SE$ ($10^8 \times M^{-1}$) | $R \pm SE$ (fmol/ 10^6 cells) | |
| T-47D | 0 | 4 | ^b | ^b | 0.93 ± 0.12 | 55.58 ± 7.09 | 0.1 |
| | 3.8×10^{-9} | 3 | ^b | ^b | 1.33 ± 0.26 | 38.30 ± 7.00 | 0.1 |
| | 1.7×10^{-7} | 3 | ^b | ^b | 1.02 ± 0.19 | 46.00 ± 8.32 | 0.1 |
| MCF-7 | 0 | 3 | 3.38 ± 2.05 | 1.93 ± 1.10 | 0.84 ± 0.24 | 68.04 ± 12.21 | 0.2 |
| | 3.8×10^{-9} | 3 | 2.37 ± 1.93 | 2.89 ± 2.25 | 0.54 ± 0.21 | 113.4 ± 28.59 | 0.2 |
| | 1.7×10^{-7} | 3 | 2.33 ± 1.64 | 2.97 ± 2.12 | 0.76 ± 0.27 | 78.02 ± 15.62 | 0.2 |
| HCT-8 | 0 | 5 | 4.15 ± 1.02 | 5.60 ± 0.97 | 0.85 ± 0.32 | 34.13 ± 7.10 | 0.1 |
| | 3.8×10^{-9} | 4 | 3.82 ± 0.87 | 3.83 ± 0.73 | 1.15 ± 0.40 | 21.22 ± 3.70 | 0.1 |
| | 1.7×10^{-8} | 4 | 6.03 ± 3.43 | 2.83 ± 1.61 | 4.27 ± 2.57 | 11.47 ± 1.98 ^c | 0.1 |
| | 1.7×10^{-7} | 5 | 4.62 ± 1.25 | 5.01 ± 1.01 | 1.25 ± 0.70 | 18.99 ± 5.48 | 0.1 |

^a K_a , binding affinity; R , binding site concentration.

^b Data fit best a single binding site model.

^c Significant difference from zero insulin concentration ($P < 0.05$).

Table 2 Equilibrium insulin binding to HCT-8 cells before and after receptor down regulation using Ligand analyses of pooled data fitted to a one-site binding model

| Insulin concentration used for down regulation (M) | No. of experiments | $K_a \pm SE$ ($10^9 \times M^{-1}$) | $R \pm SE$ (fmol/ 10^6 cells) | N (% bound) |
|--|--------------------|---------------------------------------|---------------------------------|---------------|
| 0 | 5 | 1.58 ± 0.25 | 12.95 ± 1.20 | 0.1 |
| 3.8×10^{-9} | 4 | 1.43 ± 0.20 | 9.54 ± 0.82 ^b | 0.1 |
| 1.7×10^{-8} | 4 | 1.93 ± 0.27 | 9.09 ± 0.74 ^b | 0.1 |
| 1.7×10^{-7} | 5 | 2.16 ± 0.33 | 10.59 ± 0.88 | 0.1 |

^a K_a , binding affinity; R , binding site concentration; N , nonspecific binding.

^b Significant difference from zero insulin concentration by the Tukey Kramer test ($P < 0.05$).

Table 3 Dose response curves for insulin-stimulated leucine incorporation into protein over 7 h

| Cell | Down regulation | % response above basal at insulin concentration (M) (mean ± SD) ($n = 3$) | | | |
|-------|-----------------|---|-----------|-----------|-----------|
| | | 10^{-9} | 10^{-8} | 10^{-7} | 10^{-6} |
| MCF-7 | - | 7 ± 8 | 18 ± 7 | 27 ± 18 | 40 ± 12 |
| | + | 15 ± 11 | 15 ± 9 | 42 ± 24 | 52 ± 23 |
| T-47D | - | 13 ± 13 | 14 ± 6 | 42 ± 9 | 51 ± 10 |
| | + | 8 ± 7 | 13 ± 5 | 29 ± 1 | 37 ± 14 |
| HCT-8 | - | 15 ± 5 | 14 ± 8 | 19 ± 5 | 23 ± 9 |
| | + | 17 ± 13 | 21 ± 9 | 21 ± 14 | 33 ± 10 |

sponse to insulin in the same cells. The basal rate of incorporation of [³H]leucine into protein over 7 h was not significantly different between control cells and those preexposed to insulin. The means and SDs for the dose-response curves for insulin-stimulated [³H]leucine incorporation into protein are shown in Table 3. Attempted down regulation of receptors by prior exposure to insulin did not significantly alter the responsiveness to insulin in any of the tumor cell lines. These results may be contrasted to normal cells where down regulation of insulin receptors is associated with a reduction in the subsequent biological responsiveness of cells to insulin (9, 10).

The possibility was investigated that the practice of growing breast tumor cell lines with supraphysiological concentrations of insulin (MCF-7 and T-47D) and hydrocortisone (T-47D) could influence the subsequent biological response to insulin even though cells were deprived of insulin, hydrocortisone, and FCS for 24 h prior to all experiments. However, when the two breast tumor cell lines were grown in the presence of MEM and 5% FCS alone, the responsiveness to insulin (before and after receptor down regulation) was not significantly altered (data not shown).

DISCUSSION

This study demonstrates that down regulation of insulin receptors by exogenous insulin is virtually absent in two human breast tumor cell lines and is also clearly different from normal cells in a human colon tumor cell line. These findings are in agreement with and expand previous results obtained in these cells using a limited range of insulin concentrations over a fixed time period (7). Normal cells show significant reduction in hormone binding after several hours of exposure to as little as 10^{-9} M insulin (1, 7).

The paradoxical resistance of insulin receptors in HCT-8 cells to down regulation with exposure to increasing amounts of insulin was associated with increased receptor binding affinity for insulin. HCT-8 cells appear to be very sensitive to down regulation at mildly supraphysiological insulin concentrations where loss of binding results from an apparent reduction in receptor number with little change in binding affinity. However, increasing insulin concentrations induce a resistance to down regulation in HCT-8 cells. This is associated with an increased receptor binding affinity, although there is a persisting reduction in receptor number. The phenomenon is similar to that seen in the Fao cell line derived from the Reuber H35 rat hepatoma, where resistance of insulin receptors to down regulation also occurs and appears to be a consequence of increased receptor affinity (18). The magnitude of change in binding affinity observed in the present study is, however, less than that observed in Fao cells. Changes of insulin receptor binding affinity have also been described in rat chondrosarcoma cells by Stevens *et al.* (19), where insulin induced an increase in high-affinity insulin binding.

Insulin-stimulated protein synthesis was assessed in the present study to determine whether differences in receptor down regulation between the breast tumor cell lines and the colon tumor cell line were reflected in corresponding changes in biological responsiveness to insulin. Down regulation had no effect on the responsiveness of tumor cells to insulin. The breast tumor cells were resistant to down regulation and therefore little change in responsiveness to insulin would have been expected. However, insulin binding to the colon tumor cell line, HCT-8, decreased by 25% after 4 h exposure to 1.7×10^{-8} M insulin and therefore a small loss in responsiveness to insulin could have been expected. During the 7 h of insulin stimulation used to assess [³H]leucine incorporation into protein, some ongoing down regulation of receptors may have occurred especially in control HCT-8 cells. However, the response to insulin was similar when measured at either 2 (data not shown)

or 7 h, suggesting that intraassay receptor down regulation did not influence the pattern of biological response.

The present results indicate that two human breast tumor cell lines are resistant to insulin-induced receptor down regulation and do not change their biological response to insulin following prolonged hormone exposure. Insulin induces a resistance to receptor down regulation in a human colon tumor cell line, although this is not reflected in an altered biological response to insulin. It is possible that the abnormality of receptor down regulation in such cells may contribute to the abnormal pattern of cell growth typical of malignant transformation.

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