Growth-promoting Effect, Biological Activity, and Binding of Insulin in Human Intestinal Cancer Cells in Culture

Jean-Pierre Cezard, Marie-Elisabeth Forgue-Lafitte, Marie-Claude Chamblier, and Gabriel E. Rosselin

ABSTRACT

The biological action and binding of insulin were tested in two human intestinal cancer cell lines originating from the duodenum (HUTU 80) and the colon (HT 29).

After serum deprivation for 24 hr, insulin stimulated cell division and the incorporation of labeled precursors into RNA, protein, and DNA for both cell lines. The action on the RNA and protein was rapid and significantly different (1.5 to 2 times that of control) 1 hr after adding insulin. These effects were dose dependent, present at physiological concentration in vivo (10⁻¹⁰ M), and independent of the transport of precursors. For thymidine incorporation, the stimulation was delayed up to 8 hr and culminated with cell division 20 hr later.

As previously shown for HT 29, HUTU 80 cells exhibited insulin-specific binding sites. Binding of ¹²⁵I-insulin was saturable; reversible; and time, temperature, and pH dependent. Scatchard analysis of the binding data of the two cell lines gave curvilinear plots. Assuming the presence of two independent binding sites, the high-affinity constants were 6 to 8 x 10⁶ M⁻¹, and the number of high-affinity receptors was similar and accounted for 2000 to 3000 receptors/cell.

For both cell lines, the effect of insulin on protein and RNA synthesis was significantly different from control at 1 hr when binding reached a maximum at 37°C. The biological action of insulin on growth and macromolecular synthesis was dose dependent and maximum at about 10⁻⁸ M insulin, which corresponds to 70% displacement of ¹²⁵I-insulin binding. Furthermore, the binding and the biological action of proinsulin were about 2% that of native insulin in the two cell lines studied.

These results show that insulin acts as a growth factor for these two cell lines and that these effects are probably mediated by the interaction of insulin with specific receptors.

INTRODUCTION

Insulin as a factor of growth (20, 24, 27, 37, 50) and differentiation (3, 6, 16, 27, 34, 49) has been studied in a large variety of target tissues such as fat (6, 8, 16, 27, 32, 42), fibroblast (24, 25, 37, 50), liver (3, 51), and mammary cells (22, 23, 49). Furthermore, with other hormones and external factors, insulin is a determinant for the growth and the adaptation of many mammalian cells to the environmental medium in culture (20, 21, 45).

Likewise, hormones have been involved in the development of hormone-associated cancers (18, 36, 41, 48). In culture of hepatoma (4) and human breast cancer cells (38, 39), the biological action of insulin has been correlated with the presence of specific receptors. However, the role of insulin in the development of these tumors remains conjectural in vivo.

Until recently, the biological activity of insulin on the gut has been conflicting (2, 5, 15, 26, 35). The variable results could be related to the fact that most of the experiments were performed in vivo and studied the uptake of glucose and amino acids (1, 2, 5, 7, 15, 26), which does not appear to be regulated in the gut by insulin. However, the possible action of insulin on the ontogenesis of sucrase in the small intestine of the sucking mouse (35) and the recent identification of specific receptors of insulin in the rat enterocyte (13) have emphasized the possible direct action of insulin on the development of the digestive tract. Furthermore, the presence of insulin-binding sites in a human tumoral cell line from the colon (12) suggested also an insulin dependence of human digestive cancers.

The type of this work was to determine in 2 human tumoral intestinal cell lines a biological action of insulin on cell growth and thymidine incorporation into DNA and to correlate the presence of specific receptors of the hormone with transport of nutrients and macromolecular synthesis.

The 2 cell lines used were isolated from human adenocarcinoma of the duodenum (HUTU 80) and the column (HT 29) by Fogh et al. (11).

MATERIALS AND METHODS

Chemicals. Pork monocomponent and crystalline beef insulin, pork proinsulin, and glucagon were purchased from Novo, Bagsvaerd, Denmark. Dulbecco’s modified Eagle’s Medium 196 G, FCS (Ca²⁺ and Mg²⁺ free), PBS, and 0.05% trypsin-0.02% sodium EDTA solution were obtained from Grand Island Biological Co., Paisley, Scotland. BSA Fraction V was obtained from Miles Laboratories, Inc., Elkhart, Ind. Culture flasks and sterile plastic laboratory wares were from Nunclon, Kastrup, Denmark. L-[U⁻¹⁴C]leucine (300 mCi/mmol), [5,6⁻¹⁴C]thymidine (45 Ci/mmol), [methy¹⁴C]uridine (52 Ci/mmol), and ¹²⁵I IMS 300 were purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England. L-Leucine, uridine, thymidine, and fatty acid-free BSA were from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade and were purchased from Merck, Darmstadt, West Germany.

Cell Culture. HUTU 80 and HT 29 cells were routinely grown in plastic culture flasks in Dulbecco’s modified Eagle’s medium containing penicillin (100 µg/ml) and streptomycin (100 µg/
ml) supplemented with 10% FCS and equilibrated with 10% CO₂ in air. The cells were passaged at subconfluence using a mixture of 0.05% trypsin and 0.02% EDTA, centrifuged, and washed with the culture medium before seeding. The medium was changed every 48 hr. Because the presence of serum in washed with the culture medium before seeding. The medium CO₂ in air. The cells were passaged at subconfluence using a then precipitated with 1 ml of 10% trichloroacetic acid. Acid-soluble intracellular pools of labeled precursors. Cells were then harvested by gentle scraping with a rubber policeman, collected by centrifugation, and resuspended in fresh PBS. A small aliquot was taken for dioactive precursors. Cells were then harvested by centrifugation as described previously (17), and the radioactivity was determined in a Packard liquid scintillation counter. Acid-soluble intracellular pools of labeled precursors were measured by counting the supernatant collected after the filtration of the acid-insoluble material.

**Binding Assay of Insulin.** Binding assays of insulin were performed on cells deprived of serum for 24 hr and harvested with 0.02% EDTA according to methods described previously for the HT 29 cell line (12) and the rat enterocyte (13). Pork monocomponent insulin was iodinated by the chloramine-T method and purified on Sephadex G-50 (25). Cells (0.2 × 10⁸ to 4.10⁶/ml) were incubated in duplicate samples with [¹²⁵I]insulin (3.2 × 10⁻¹¹ to 1.6 × 10⁻¹⁰ M) in 0.5 ml Krebs-Ringer phosphate buffer (pH 7.8) containing 1% fatty acid-free BSA. Other conditions are described in the chart legends. After incubation, insulin bound to cells was separated by centrifugation as described previously (17), and the radioactivity was determined in a γ spectrometer. Data are reported as specific binding; this is obtained by subtracting from the total the nonspecific binding, i.e., the amount of labeled insulin that is not displaced by an excess (1.6 × 10⁵ M) of unlabeled insulin.

The degradation of [¹²⁵I]insulin in the incubation medium was tested by its ability to bind rat liver plasma membranes as described previously (14). Viability of the cells at the end of the experiments was verified by trypan blue exclusion (>95%).

**Statistical Methods.** Numerical results are expressed as the mean ± S.E. of triplicate determinations or the mean of duplicate determinations (binding data). Binding experiments were realized in triplicate, and results varied by less than 10%. Regression lines were calculated by the method of the least squares. The significance of the difference between paired numerical data or between the slopes of regression lines was calculated by Student’s t test.

**RESULTS**

**Effect of Insulin on Growth.** Insulin stimulated the growth of HUTU 80 and HT 29 (Chart 1) in a dose-dependent manner. A significant stimulation was noted at a dose as low as 10⁻¹⁰ M in the 2 cell lines. A positive maintenance effect on cell number was observed at a concentration of 10⁻¹² M insulin in the HT 29 cell line. Insulin at a 10⁻⁸ M concentration as compared to 10⁻⁶ M elicited no further effect on the growth of HT 29 and only a slight additional effect on the growth of HUTU 80. Insulin exhibited a lower effect than FCS in promoting the growth of cells. For example, the stimulation of 10⁻⁶ M insulin at 24 hr accounted for 35 to 45% of the action of 10% FCS (Table 1). The addition of 10⁻⁶ M insulin to 10% FCS did not increase the growth of both cell lines over that observed with 10% FCS alone (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of HUTU 80 cells/flask</th>
<th>No. of HT 29 cells/flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>298,000 ± 19,000⁶</td>
<td>966,000 ± 16,000⁶</td>
</tr>
<tr>
<td>10⁻⁶ M Insulin</td>
<td>626,000 ± 16,000⁶</td>
<td>1,460,000 ± 61,000⁶</td>
</tr>
<tr>
<td>10% FCS</td>
<td>910,000 ± 41,000⁶</td>
<td>2,040,000 ± 80,000⁶</td>
</tr>
<tr>
<td>10% FCS + 10⁻⁶ M insulin</td>
<td>860,000 ± 120,000⁶</td>
<td>1,895,000 ± 35,000⁶</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of triplicate determinations.
  b Significantly different from control (p < 0.01).
  c Not significantly different from 10% FCS.
extracellular pool of unlabeled leucine of the incubation medium; in contrast, [3H]uridine represented the total extracellular pool. A decrease of 50% of the extracellular pool of [3H]uridine was noted after 3 hr of incubation, but no difference was seen in the L-[14C]leucine extracellular pool. The stimulation of insulin was dose dependent and significantly different from control at any concentration tested (Chart 3). No further stimulation of incorporation was noticed for uridine and leucine above 10^-8 M insulin. Proinsulin (10^-10 to 10^-6 M) stimulated the incorporation of leucine and uridine, and the stimulation was about 2% that of native insulin (data not shown).

Thymidine incorporation was also stimulated by insulin (Chart 4), but an 8-hr lag period was observed. Maximum stimulation was observed between 8 and 16 hr after adding insulin and the labeled precursor. Cell division appeared in insulin-stimulated cells between the 16th and the 24th hr after the beginning of the experiments. These results suggested an increase in the number of cells entering S phase after the eighth hr and then mitosis and division after the 16th hr as compared to controls.

The intracellular acid-soluble pools of labeled precursors were also measured during the precursor incorporation studies. There was no difference as compared to controls for the pools of labeled leucine and uridine. However, an increase of the [14C]thymidine intracellular acid-soluble pool was noticed in the insulin-stimulated cells before the incorporation of thymidine into DNA. A significant difference (p < 0.05) was present 4 and 8 hr after adding the hormone (data not shown).

**Insulin Binding Studies.** Insulin binding to HUTU 80 cells gave results similar to those obtained previously on HT 29 cells (12). As shown in Chart 5, insulin binding was saturable, reversible, and time and temperature dependent. The absence of steady state of binding at 37°C might be explained at least in part by the high rate of degradation of 125I-insulin by detached HUTU 80 cells (Chart 6). Despite the lower degradation by attached HUTU 80 cells in medium culture (Chart 6), these results contrasted with the absence of degradation of insulin by HT 29 cells in culture medium (12). 125I-Insulin binding was linearly related to the number of cells between 0.2 and 4 x 10^6 cells/ml and was pH dependent with a maximum binding between pH 7.8 and pH 8.

The concentration dependence of insulin binding was determined on both cell lines by adding increasing concentration of unlabeled insulin (10^-10 to 10^-7 M) to a fixed concentration of 125I-insulin (4 x 10^-11 M) in the incubation mixture under optimal conditions of apparent equilibrium. For 3 hr of incubation at 15°C, nonspecific binding was 20% of the total binding and degradation of 125I-insulin was 20% (Chart 6). Native insulin reduced tracer binding in a continuous dose-dependent fashion, and the half-maximum binding dose was obtained at 5 to 2 x 10^-8 M unlabeled insulin (A) or without insulin (A) were added at Time 0. Cells were harvested at the times shown. Points, mean – S.E. (bars) of triplicate determinations.

**Chart 5.** Dose effects of insulin on the rate of L-leucine (O) and uridine incorporation in HUTU 80 (A) and HT 29 (B) cell lines. [3H]Uridine [5 yCi (A), 2.5 yCi (B)] with 10^-6 M insulin (A) or without insulin (B) and [3H]leucine (5 yCi (A), 2.5 yCi (B)] with 10^-6 M insulin (C) or without insulin (D) were added at Time 0. Cells were harvested at the times shown. Points, mean – S.E. (bars) of triplicate determinations.

**Chart 6.** Time course of 125I-insulin binding to cell lines. Points, mean – S.E. (bars) of triplicate determinations.
Insulin Action and Binding in Human Gut Cancer Cell Lines

Chart 5. A, time course of insulin binding to HUTU 80 cell line. $^{125}$I-insulin ($4 \times 10^{-11}$ M) was incubated as indicated in "Materials and Methods" with 1.5 x $10^6$ cells at 37° (•) and 15° (O) for the time indicated on the abscissa. The resulting binding curve represents specific binding ($\text{specific binding} = \text{total binding} - \text{nonspecific binding}$). Nonspecific binding did not change appreciably during the experiment and amounted to 40% at 37° and 20% at 15° of total labeled insulin bound. B. dissociation of bound $^{125}$I-insulin to HUTU 80 cell line. Cells ($0.8 \times 10^6$) were incubated for 3 hr at 15° with $8 \times 10^{-5}$ M $^{125}$I-insulin, and the specific binding was determined. This corresponds to 100% on the ordinate. To duplicate sample, $1.6 \times 10^{-5}$ M unlabeled insulin was added, and the incubation was continued for various lengths of time as indicated on the abscissa. At these various times, specific binding of $^{125}$I-insulin was determined.

Chart 6. Time course of degradation of $^{125}$I-insulin on HUTU 80 cell line. Attached cells at 37° or detached cells at 37° and 15° (1.5 x $10^6$) were incubated with $^{125}$I-insulin ($4 \times 10^{-11}$ M) at the times and temperatures indicated and centrifuged. The supernatants were then assayed for insulin integrity as described in "Materials and Methods." Attached cells at 37° and 15° showed a slower degradation of insulin than the detached cells at 37°. The degradation rate was calculated as the percent of initial binding and is shown in the graph.

Chart 7. Concentration dependence of insulin binding (one typical experiment) on HUTU 80 (A) and HT 29 (B) cell lines. Cells ($0.7 \times 10^6$) were incubated with $10^{-10}$ to $10^{-7}$ M $^{125}$I-insulin in the absence (100%, ordinate) and presence of increasing concentrations of unlabeled insulin for 3 hr at pH 7.8 and 15°. Inset, Scatchard plot obtained from the binding data.

The specificity of binding was tested in competition experiments between $^{125}$I-insulin ($4 \times 10^{-11}$ M), unlabeled insulin ($10^{-10}$ to $10^{-7}$ M), proinsulin ($10^{-9}$ to $10^{-6}$ M), and glucagon, an unrelated peptide. Fifty % of the labeled hormone (half-maximum binding dose) was displaced by $5 \times 10^{-8}$ M proinsulin for HT 29 and $10^{-7}$ M for HUTU 80. When these results are compared to the half-maximum binding dose obtained with insulin, it indicates that the ability of proinsulin to bind cells is 4% that of native insulin for HT 29 and 2% (not significantly different) for HUTU 80. Glucagon, an unrelated peptide ($10^{-7}$ to $10^{-6}$ M), did not compete with $^{125}$I-insulin for the binding sites of either cell line.

DISCUSSION

The availability of cell lines maintained in culture has facilitated the study of hormone actions. In particular, it allowed the demonstration of a direct interaction between the hormone and the cell and its biological effect at physiological dose, and it permitted separation of this action from the effect of other hormones which could influence or modulate the same pathway.

This study demonstrates that insulin has a biological action at physiological concentration in vivo ($10^{-10}$ M) on 2 human tumoral intestinal cell lines in culture. This effect has been observed on cell division and incorporation of precursors into macromolecules, and is related to the presence of receptors specific to insulin.

As do most mammalian cells (20, 21) which need insulin to grow in culture in defined synthetic medium, these 2 cell lines respond to insulin as a growth factor. However, the limited stimulation obtained with insulin as compared to FCS and the conditions under which growth stimulation has been observed, i.e., deprivation of serum for 24 hr which results in a quiescent phase in G0-G1 of the cell cycle (46), suggested that other growth factors, contained in FCS, are necessary to assure a normal growth in culture and that insulin might act during a critical phase of the cell cycle.
The mechanisms by which insulin stimulates growth and macromolecular synthesis after binding to its receptor remain unknown. However, it appears that it is not the result of an increase of uridine and amino acid uptake, as has been found for human breast cancer cells (28). It seems also not to be the result of nonspecific stimulation of cell metabolism by enhanced glucose availability. Preliminary studies on d-methyl glucose and aminoobutyric acid uptakes by these 2 cell lines following the techniques of Lecam and Freychet (31) and Kimmich (28) exhibit no significant difference with the insulin-stimulated cells as compared to the controls. This fact may be related to the absence of sensitivity to insulin of the glucose and amino acid carriers, as has been suggested for the glucose carrier in the enterocyte (2, 26). Furthermore, it suggests that the first action of insulin occurs at a site other than membrane transport (52, 53). In contrast, the increase in thymidine pool size observed prior to the increase of DNA synthesis might account at least for part of the insulin effect on the rate of incorporation of thymidine into DNA. This effect, observed by Osborne et al. (39) on human breast cancer cell lines, could either be a stimulation of the uptake rate of thymidine or secondary to the stimulation of thymidine kinase activity and accumulation of TMP. At any rate, the thymidine incorporation studies reflect an increase in the synthesis of DNA in the insulin-stimulated cells because this increase is followed by cell division.

The sequence of events in the action of insulin appears to be early stimulation of protein and RNA synthesis followed 8 to 10 hr later by enhanced DNA synthesis culminating in cell division and growth 20 to 24 hr later. Besides this action of insulin on growth, it was of interest to investigate whether the synthesis of specific proteins involved in a differentiation process might correspond to 70% displacement of insulin binding. Furthermore, the binding and the biological action of proinsulin on protein and RNA were about 2% that of native insulin for both cell lines. On the other hand, no relation between the biological effect and the degradation of insulin was observed in contrast to the finding of Osborne et al. (39) for human breast cancer cell lines. It remains that these results strongly support the argument that the biological action of insulin is mediated by its interaction with its specific receptors. Therefore, it appears that this model offers unique advantages for the study of the trophic action of insulin on the digestive tract. Although no extrapolation to the in vivo situation could be done, these results suggest a possible action of insulin on human gut cancer.

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REFERENCES


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