Rapid Publication

# Insulin Suppresses Rat Growth Hormone Messenger Ribonucleic Acid Levels in Rat Pituitary Tumor Cells

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**SUMMARY**

**Insulin has been shown to suppress growth hormone (GH) secretion by rat pituitary tumor cells ($GH_3$) independently of glucose utilization. The effects of physiologic doses of insulin were therefore tested on cytoplasmic levels of GH messenger ribonucleic acid (mRNA) sequences. Insulin (3.5 nM) treatment of cells for 5 days suppressed the hybridization of cytoplasmic GH mRNA with $^{32}$P-cDNA for rGH by 50%. The three- to fourfold induction of cytoplasmic GH mRNA by hydrocortisone (1 μM) was also suppressed by insulin (3.5 nM) by 40%. The results show a direct suppression of cytoplasmic rGH mRNA concentration by physiologic doses of insulin. These findings may be due to either decreased rate of GH gene transcription, increased intracellular breakdown, or decreased nuclear-cytoplasmic transport of GH mRNA caused by insulin.**

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Insulin regulates cellular gene expression and modulates specific messenger ribonucleic acid (mRNA) levels in several cells.[1, 11] These actions of insulin are independent of its effects on glucose or amino acid transport.[1] Insulin was shown to regulate pituitary carbohydrate metabolism[12] and protein synthesis in vitro[13] and insulin receptors have been characterized on normal pituitary tissue[14, 16] and on GH rat pituitary tumor cells.[17, 19] Reports that hypothalamic concentrations of insulin were significantly higher than in other rat brain regions[20] are also consistent with a role for insulin in regulation of pituitary function. Direct modulation of pituitary GH expression by insulin and somatomedin peptides (insulin-like growth factor, IGF) has been studied in several in vitro[21, 25] and in vivo[26,27] systems. We have recently demonstrated that insulin inhibits the in vitro secretion of GH by rat pituitary tumor cells ($GH_3$) and also inhibited both the hydrocortisone[25] and $T_3$-induced[28] stimulation of GH secretion. These effects were observed at insulin doses within the physiologic range, and were independent of glucose utilization. These studies are all consistent with a specific role for insulin in cellular regulation of GH secretion. We therefore undertook to further define the action of insulin on $GH_3$ cells by measuring relative levels of cytoplasmic GH mRNA sequences. This allowed us to assess whether insulin altered the specific rGH mRNA content of these cells, and to gain further insight into relative perturbations of mRNA by insulin during hydrocortisone-induced mRNA stimulation. The results show that insulin suppresses both the basal and hydrocortisone-induced GH mRNA content in these cells.

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## METHODS

**Cells.** Rat pituitary tumor cells ($GH_3$), secreting GH and PRL,[29] were cultured in monolayer as previously described.[25] Culture medium used was Ham's F10 enriched with fetal calf serum (2.5%), horse serum (15%), glutamine (5 mM), and antibiotics. Insulin was undetectable (<5 μU/ml) in this medium. Before seeding, cells were pretreated with either purified semisynthetic human insulin (Squibb Novo, Princeton, New Jersey) or vehicle for 48 h. Cells were seeded in multiwell plates (35 mm diameter, Costar, Cambridge, Massachusetts) containing 2 ml of medium enriched with the indicated hormones and incubated for 5 days. As 40% of added $^{125}$I-insulin was shown to undergo degradation during 72 h of incubation under identical conditions,[25] 40% of the initial insulin dose was replenished to maintain the initial medium insulin concentration. At the end of the experiments, medium was aspirated and cells taken up aseptically for extraction of cytoplasmic RNA. An aliquot of cells was removed for counting before RNA extraction.

**Nick translation.** The cloned cDNA insert for rat GH (rGH-cDNA 800)[30] was kindly provided by Drs. John Baxter and Norman Eberhardt, University of California, San Francisco. Deoxycytidine-5′-triphosphate [-$^{32}$P] (sp act > 600 Ci/mmol) was obtained from New England Nuclear (Boston, Massa-

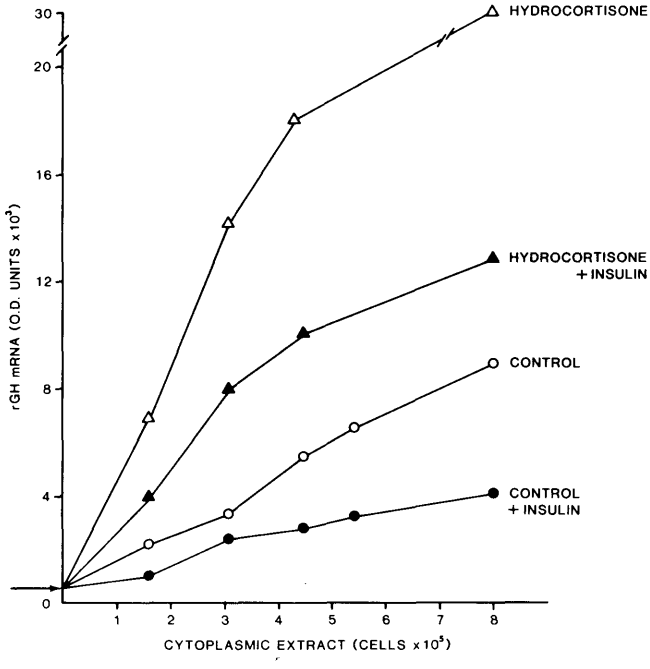


**FIGURE 1. Cells were grown for 5 days without added hormones (controls) or hydrocortisone (1 μM) and/or insulin (3.5 nM). Cells from six multiwells were pooled, cytoplasm was extracted, and relative levels of GH were measured by densitometer scanning of a cytoplasmic dot blot autoradiograph. Each point represents the mean integrated reading obtained from each cell pool in three independent experiments. Arrow indicates level of hybridization signal obtained from cytoplasmic extracts (4.5 × $10^5$ cells) treated with RNAse.**

chusetts) and nick translation[31] of 0.5 μg rGH cDNA was performed as described using DNA polymerase (Pol 1, 0.6 U/μl) and DNAse (0.2 U/ml) with commercial nick translation reagents (New England Nuclear). The radiolabeled rGH-cDNA probe was purified by eluting over a Sephadex G-100 column with 10 mM EDTA, pH 7.5, and mixed with 0.1 M sodium acetate, 2 mM magnesium acetate, and 2.5 vol ethanol (4°C). The DNA was precipitated for 14 h at −20°C, centrifuged at 12,000 × *g* for 10 min, and resuspended in distilled water (sp act ≈ $10^8$ dpm/μg DNA).

**Cytoplasmic extraction.** RNA was extracted aseptically from the cells, essentially as described.[32] Briefly, cells were pelleted and resuspended in 1 ml phosphate-buffered saline, repelleted at 15,000 × *g* for 15 s, and resuspended in ice-cold 10 mM Tris, 1 mM EDTA (pH 7). Cells were lysed in 5% Nonidet P-40 and nuclei pelleted at 15,000 × *g* for 2.5 min. The supernatant (50 μl) was mixed with 0.15 M sodium chloride and 0.015 M sodium citrate (30 μl) and 37% (wt/wt) formaldehyde (20 μl) at 60°C for 15 min, followed by storage of the extracts at −70°C until assayed. For mRNA analysis, sample extracts were diluted with 15 × sodium chloride–sodium citrate in 96-well microtiter plate to yield a final total volume of 150 μl.[32,33]

**Dot hybridization.** Prewetted nitrocellulose filter paper (BA 85, 0.45 μM), supported on no. 470 paper, was mounted in a 96-hole Minifold microsample filtration vacuum manifold (Schleicher and Schuell, Keene, New Hampshire). Cytoplasmic RNA was immobilized by spotting 100 μl of extract sample on the nitrocellulose, and after vacuum filtering, the paper was baked for 2 h at 80°C.[33] The paper was prehybridized at 42°C as described,[32] except that 5 × Denhardt's reagent and 200 μg/ml carrier salmon sperm DNA were used in the buffer, and the incubation was for 8–12 h. After the prehybridization, buffer was discarded, the labeled rGH-cDNA probe (denatured by boiling) was added with the hybridization buffer, which also contained 10% dextran sulfate, and the incubation continued for 48 h. The nitrocellulose paper was washed as described,[32] except that 0.1% sodium dodecyl sulfate was used and the second pair of washes was performed at 50°C for a total of 1 h. After exposure of the paper to x-ray film, autoradiographic dots were quantified by densitometric scanning, using an EC Apparatus Corporation Densitometer (St. Petersburg, Florida) and Hewlett Packard (Palo Alto, California) 3390A Integrator.

**Controls.** Some $GH_3$ cell cytoplasmic extracts were initially treated with bovine pancreatic ribonuclease A (0.1 μg/ml, Sigma, St. Louis, Missouri) in 10 mM Tris, 1 mM EDTA, pH 7.5, for 1 h at 37°C to confirm the RNAse sensitivity of the hybridization. For negative controls, cytoplasmic extracts of non–hormone-secreting mouse L-cell fibroblasts were performed in identical fashion to the $GH_3$ cell extracts and these were blotted on the same nitrocellulose filter.

## RESULTS

Autoradiography of the nitrocellulose sheets revealed that the cDNA probe interacted only with portions of the filter dotted with mRNA extracts. The intensity of the hybridization varied in relation to the total number of $GH_3$ cells extracted,

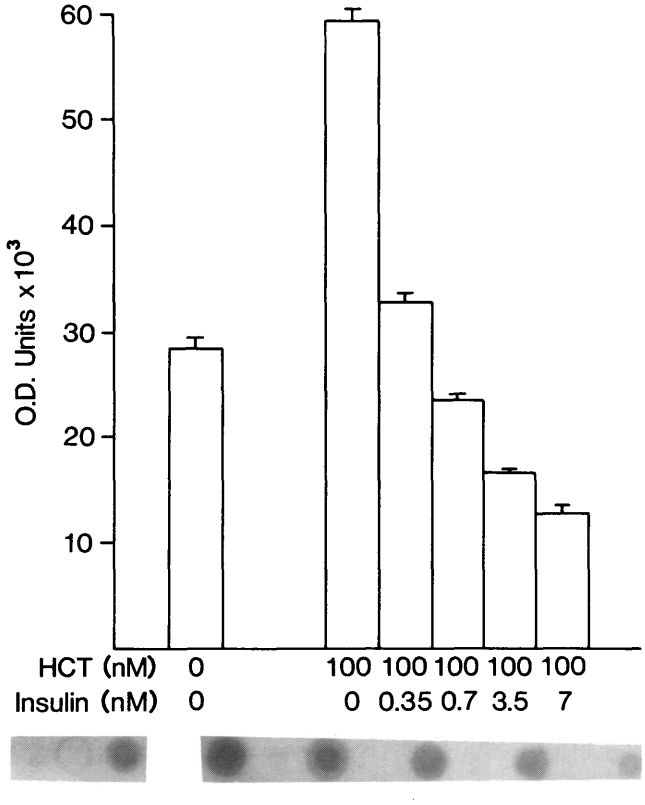


**FIGURE 2. Response of cytoplasmic GH mRNA content to varying doses of insulin. Cells were incubated for 5 days with hydrocortisone (HCT, 100 nM) and varying doses of insulin. Autoradiographs of cytoplasmic dot blots were scanned by densitometer. Insert shows representative autoradiograph (from left to right: RNAse-treated extract, control, and HCT with 0–7 nM insulin, respectively).**

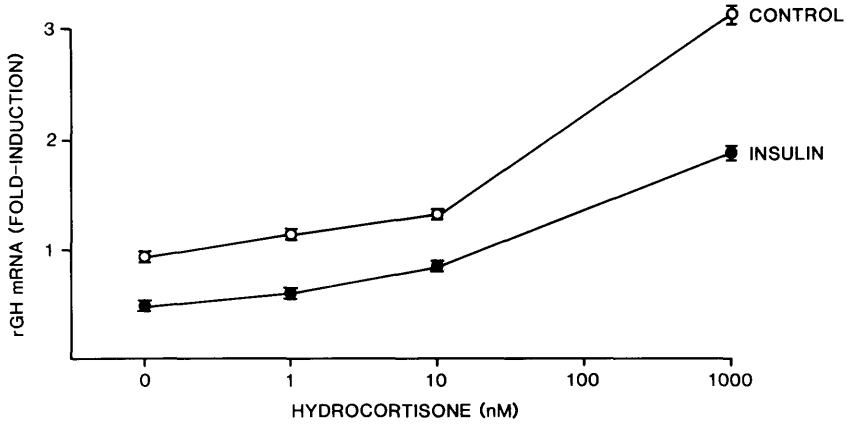


**FIGURE 3. Induction of GH mRNA sequences by hydrocortisone during 5 days incubation was determined in the absence (control) or presence of insulin (3.5 nM). Each point represents mean integrated optical density of pooled cell extracts ($4.9 \times 10^5$ cells) in three separate experiments (± SEM).**

and the hybridization response diminished appropriately as the cytoplasmic extracts were diluted (Figure 1). Treatment of cells with insulin (3.5 nM) for 5 days clearly inhibited the cytoplasmic GH mRNA hybridization and the intensities of the insulin-treated cytoplasmic extracts were also proportional to the amount of cytoplasm dotted. Insulin inhibited the relative GH mRNA content in cells induced by hydrocortisone (1 μM). The hybridization was sensitive to pancreatic ribonuclease, and cell extracts treated with the enzyme yielded very low hybridization intensities (Figure 1). Cytoplasmic extracts of mouse L-cells (negative controls) consistently yielded negligible intensities that were indistinguishable from the x-ray film background.

**Effects of rGH mRNA on hydrocortisone induction.** Treatment of the cells with insulin resulted in an attenuation of the GH mRNA induced by hydrocortisone (Figure 2). Incubation of the cells with insulin (0.35 nM) suppressed the GH mRNA induced by 100 nM hydrocortisone by about 50%, while higher doses of insulin actually prevented the hydrocortisone-induced stimulation of GH mRNA. The threefold induction of rGH mRNA by hydrocortisone (1 μM) was suppressed by at least 40% by insulin (3.5 nM) in three separate experiments (Figure 3).

## DISCUSSION

This study shows that physiologic doses of insulin suppress the GH mRNA content in $GH_3$ cells. Insulin also inhibits the three- to fourfold increase of GH mRNA induced by hydrocortisone by 40%. The findings are consistent with previous studies showing that similar doses of insulin inhibit the secretion of GH by these cells after a 48-h lag period.[20] Insulin did not alter $GH_3$ cell replication, and suppression of GH secretion occurred in glucose-free medium and was associated with a stimulation of PRL secretion.[25]

The specific structure of the cDNA rat GH probe used for hybridization has been fully characterized.[30] The specificity of the hybridization procedure was further confirmed by its sensitivity to ribonuclease as well as the absence of hybridization by non–GH-secreting mouse fibroblasts. The selectivity of the inhibitory effect of insulin on GH mRNA was demonstrated by the fact that PRL mRNA was stimulated by insulin in the same cells.[34] Furthermore, insulin did not suppress total cellular RNA and cell replication or affect viability of these pituitary tumor cells (data not shown).

It is unlikely that these effects of insulin were caused by activation of a somatomedin or IGF-I receptor. The dose of insulin required for half-maximal displacement of $^{125}$I-IGF I from binding sites on pituitary membrane receptors was 200 nM,[15] while 40 nM insulin caused negligible $^{125}$I-IGF displacement from pituitary receptors in another study.[16] These concentrations of insulin were clearly far higher than the doses of insulin used in this study. Previous studies have also shown that the dose of IGF-I required for maximal inhibition of GH secretion by these $GH_3$ cells is about 32 nM.[25] Furthermore, IGF-I did not reverse the hydrocortisone-induced stimulation of GH secretion, further suggesting a separate specific action for insulin on GH gene expression.[25] Nevertheless, an effect of insulin mediated by an IGF receptor cannot be excluded.

The induction of cytoplasmic GH mRNA by hydrocortisone seen in these cells confirms previous reports in which transcription of GH mRNA was shown to be stimulated both in vivo[35] and in vitro[36-38] by corticosteroids. The ability of insulin to reverse the induction of long-term in vitro GH secretion by hydrocortisone[25] and cortisol[21] suggests that insulin directly suppresses GH gene expression. Higher doses of insulin (10 μg/ml) than those used in this study also inhibited the dexamethasone-induced rGH and p16 protein synthesis by GC cells as visualized by two-dimensional gel electrophoresis.[23] Although most of the studied effects of insulin on specific mRNAs are stimulatory, insulin has been shown to inhibit transcription of the phosphoenolpyruvate carboxykinase gene.[39] A direct inhibition of rat GH gene transcription is possible, but increased intracellular breakdown of GH mRNA and decreased nuclear-cytoplasmic transport of GH mRNA caused by insulin in these cells cannot be excluded.[6]

These results are further evidence of a direct regulatory effect of insulin on GH gene expression, and suggest a specific inhibition of pituitary GH secretion by insulin.

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