Insulin Negatively Regulates Albumin mRNA at the Transcriptional and Post-transcriptional Level in Rat Hepatoma Cells*

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Treatment of cultured H4-II-E rat hepatoma cells with insulin causes a large decrease in cytoplasmic serum albumin mRNA. This effect is observed at low doses of insulin (ED50 = 2 pm), consistent with the effect being mediated by interaction of insulin with high affinity insulin receptors. The reduction in cytoplasmic albumin mRNA is first observed 8–12 h following insulin addition, and albumin mRNA continues to decrease up to 28 h following hormone addition. Northern blot analysis of purified poly(A)* RNA has indicated that insulin causes a decrease in albumin mRNA relative to total cytoplasmic poly(A)* RNA. In addition, one other specific mRNA, that encoded by the a-tubulin gene, is not decreased following insulin treatment. These results indicate that insulin induces a specific decrease in albumin mRNA. This effect is largely reversed if essential amino acids are added along with the insulin, suggesting that the insulin effect is related to limitation of the cells for essential amino acids. Insulin reduces transcription of the albumin gene 4.7-fold, as measured by nuclear transcription assays. However, this inhibition of albumin gene transcription does not fully account for the 57-fold decrease in albumin mRNA, indicating that insulin also exerts a negative effect on albumin mRNA at a post-transcriptional step.

Insulin is an anabolic hormone with a wide range of effects on metabolism, including stimulation of glycogen, protein, and lipid biosynthesis. The positive effect of insulin on net protein production is caused by a combined stimulation of protein synthesis (1–4) and inhibition of protein degradation (5). The effects of insulin on protein synthesis appear to operate at both the transcriptional and translational level. In heart and skeletal muscle (3, 4), epididymal fat cells (6), and cultured fibroblasts (7), insulin stimulates protein synthesis at the translational level by stimulating the rate of peptide chain initiation. In addition to this effect on translation, insulin exerts at least part of its effect on the synthesis of specific proteins by modulating levels of mRNA. In liver, insulin increases the level of mRNA for pyruvate kinase (8), fatty acid synthetase (9), alpha-globulin (10), serum albumin (11–13), malic enzyme (14), glucokinase (15), and an unidentified protein, p33 (16), while insulin decreases levels of mRNA for phosphoenolpyruvate carboxykinase (17–19) and carbamoyl phosphate synthetase (20). Insulin also increases levels of amylase mRNA in the excocrine pancreas (21). Finally, in differentiated 3T3 adipocytes, insulin increases the level of mRNA for glyceraldehyde-3-phosphate dehydrogenase (22), and insulin in combination with dexamethasone and methylisobutylxanthine increases the levels of mRNA for pyruvate carboxylase in differentiating 3T3-L1 preadipocytes (23).

Very little is known at present about the molecular mechanism by which insulin exerts its effects on mRNA production. A change in mRNA levels could theoretically result from an altered rate of transcription, processing of transcripts, transport of mRNA from the nucleus to the cytoplasm, or a change in the rate of mRNA turnover. A direct effect of insulin on transcription has been demonstrated only in the case of phosphoeno-pyruvate carboxykinase (17–19) and the unidentified p33 protein of liver cells (16). Essentially nothing is known at the molecular level about the mechanism by which insulin regulates transcription.

In the present study, we have examined the effect of insulin on the production of mRNA for serum albumin in cultured H4-II-E rat hepatoma cells (H4 cells) which have been used widely as a model system for studies on insulin action (16–19, 24–28). Insulin has been reported previously to exert a positive effect on production of albumin mRNA in diabetic rats (11, 12) and insulin exerts a positive effect on albumin production in short term primary hepatocyte cultures (13). Surprisingly, we find that insulin specifically decreases the level of albumin mRNA and inhibits albumin gene transcription in the H4 cells.

MATERIALS AND METHODS

Cells and Cell Culture—The H4-II-E rat hepatoma cell line (H4 cell line) was obtained from Dr. C. Hofmann, Veterans Administration Hospital, Hines, IL (24). Stock cultures were maintained in minimal essential medium (MEM)1 supplemented with 10% fetal bovine serum (Gibco). The F-12/Dulbecco’s modified Eagle’s medium used for serum-free incubation of the H4 cells consisted of a 1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium, 1.2 g/liter sodium bicarbonate (Gibco, tissue culture grade), 15 mM HEPES (Behring Diagnostics Ultrol grade), and 15 mM 2-aminoethanol (Sigma), adjusted to pH 7.3 (29). The medium used for serum-free experiments was made up in high-performance liquid chromatography grade water (Fisher). All media were supplemented with penicillin (71 units/ml) and streptomycin (100 mg/ml). Cells were incubated at 37 °C under a humidified atmosphere containing 95% air plus 5% CO2.

For measurement of response to hormones, the H4 cells were plated at a density of 2 × 105 cells/10-cm dish or 75-cm2 tissue culture flask in MEM plus 10% fetal bovine serum, unless otherwise indicated. After 3 days at 37 °C, the medium was aspirated and the cells were washed once with serum-free MEM. Serum-free F-12/Dulbecco’s modified Eagle’s medium (10 ml) was then added to each dish or flask

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1 The abbreviations used are: MEM, minimal essential medium; NaDso4, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
and the incubation was continued for an additional 3 days. At this time, cells had grown to form confluent monolayers. Unless otherwise indicated, hormone additions were made directly to the conditioned medium without an additional medium change. Control cultures not treated with insulin always represented cultures incubated for exactly the same total time as the insulin-treated cultures.

DNA Clones—The rat serum albumin cDNA clone pRSA13 was obtained from Dr. T. Sargent, National Institutes of Health (30). The human α-tubulin cDNA clone k1 (31) was obtained from Dr. S. Spindler, University of California, Riverside, CA. For DNA-RNA hybridizations, the DNA plasmids were radiolabeled by nick-translation using a kit obtained from Bethesda Research Laboratories.

RNA Preparation—For preparation of RNA, monolayers were washed once with phosphate-buffered saline and cells were removed with trypsin. The cell suspension was washed with phosphate-buffered saline and cells were pelleted by centrifugation. The cell pellet was lysed at 4°C with a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM MgCl₂, 0.5% Nonidet P-40, and 10 mM vanadyl-ribonucleoside complex (Bethesda Research Laboratories). Nuclei were removed by centrifugation and EDTA (final concentration 2.5 mM) and Na₂SO₄ (final concentration 0.5%) were added to the supernatant. The supernatant was extracted three times with a mixture of phenol plus chloroform plus isooctyl alcohol (24:24:1). Between the second and third extractions, EDTA was added to a final concentration of 10 mM to dissociate the residual vanadyl-ribonucleoside complexes. RNA was precipitated twice with ethanol, redissolved in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.1% Na₂SO₄ (pH 7.5), and stored at −70°C.

Poly(A)-containing mRNA was prepared by chromatography on oligo(dT)-cellulose (Bethesda Research Laboratories). Cytoplasmic RNA samples were heat-denatured and applied to the column at room temperature (23°C) in binding buffer (0.5 M LiCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.5% Na₂SO₄). The column was washed thoroughly with binding buffer, and poly(A)-containing RNA was eluted with elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% Na₂SO₄) prewarmed to 40°C. Fractions containing RNA were precipitated with ethanol, and the RNA was stored at −70°C.

DNA-RNA Hybridization—Eight serial 1:2 dilutions of RNA, beginning with 10 μg of RNA, were immobilized on nitrocellulose filters using a Hybridot apparatus (Bethesda Research Laboratories). The hybridization mixture contained 5 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, pH 7.4), Denhardt's solution, 0.5% Na₂SO₄, 0.15 mg/ml denatured salmon sperm DNA, 50% formamide, 9% dextran sulfate, and 5 × 10⁶ cpm/ml of 3²P-labeled DNA probe. Hybridizations were carried out for 16 h at 37°C. Filters were then washed 4 times with 0.1 × SSPE plus 0.1% Na₂SO₄ at 37°C, dried, and subjected to autoradiography with Kodak X-AR-5 x-ray film. Films were scanned using an LKB UltroScan XL laser densitometer.

The amount of albumin mRNA in each dilution series was quantified by performing a linear regression analysis of the logarithm of the integrated densities of spots on the autoradiogram versus dilution. Graphic analysis of the linear regression data using the computer program Graph Pad (Academic Press, Orlando, FL) confirmed that the regression lines representing the serial dilutions of different RNA samples were parallel. Relative amounts of RNA were determined by comparing x intercept values. One integrated density unit was defined arbitrarily as an X intercept value of 13.3.

Nuclear Transcription Assays—Plasmids pBR322, pRSA13, and k1 were linearized with an appropriate restriction enzyme, heat-denatured, and applied onto nitrocellulose filters using a Hybridot apparatus (Bethesda Research Laboratories). The Hybridot apparatus was used to immobilize the RNA probes by brief heating in 0.1 M NaOH, followed by neutralization with 0.1 M NaOH and 0.02 M Na₂HPO₄, pH 7.4. The RNA probes were then hybridized to the immobilized nuclear DNA in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 0.5% Na₂SO₄ (pH 7.5), and stored at −70°C.

RESULTS

To measure effects of insulin in the absence of serum, the H4 cells were incubated in serum-free medium for 72 h, insulin was added to some cultures, and levels of albumin mRNA were measured after 24 h by dot blot hybridization in control and insulin-treated cultures. The results of a representative experiment are shown in Fig. 1. Treatment of cells with insulin for 24 h caused a dose-dependent decrease in albumin mRNA levels. The average decrease in albumin mRNA produced by 100 ng/ml insulin in six independent experiments performed on different days was 57-fold. The concentration of insulin required for a 50% reduction in the level of albumin mRNA was 2 pM (Fig. 1), consistent with insulin exerting this effect by binding to high-affinity insulin receptors on the H4 cells (18, 24–26).

A time course for the effect of insulin on albumin mRNA was shown in Fig. 2. A sharp decrease in albumin mRNA occurred at 8–12 h following insulin addition. Albumin mRNA levels continued to decrease up to 28 h following insulin addition (Fig. 2).

Northern blot analysis of albumin mRNA is shown in Fig. 3. A single major band with a molecular size of approximately 2200 bases was detected with the rat albumin cDNA probe (Fig. 3a). This molecular size closely matches the predicted size of albumin mRNA (30, 34). As shown in Fig. 3a, treatment of cells cultured in medium containing 10% serum for 3 days and transferred to serum-free medium for an additional 3 days. Insulin was then added at the concentrations indicated and incubation was continued for 24 h. Cells were removed from the dishes and cytoplasmic RNA was prepared. Dot blot analysis was performed using eight serial (1:2) dilutions, beginning with 10 μg of RNA. Hybridization was performed using 3²P-labeled pRSA13 as probe. Autoradiograms of the dot blots were scanned and integrated densities of spots were analyzed using a linear regression program as described under "Materials and Methods." The zero insulin control represents the mean of results obtained with four independent RNA preparations, mean ± S.E. All other points represent the mean of results obtained with three independent RNA preparations, mean ± S.E.

FIG. 1. Effect of increasing concentrations of insulin on the level of albumin mRNA in H4 hepatoma cells. Cells were cultured in medium containing 10% serum for 3 days and transferred to serum-free medium for an additional 3 days. Insulin was then added at the concentrations indicated and incubation was continued for 24 h. Cells were removed from the dishes and cytoplasmic RNA was prepared. Dot blot analysis was performed using eight serial (1:2) dilutions, beginning with 10 μg of RNA. Hybridization was performed using ³²P-labeled pRSA13 as probe. Autoradiograms of the dot blots were scanned and integrated densities of spots were analyzed using a linear regression program as described under "Materials and Methods." The zero insulin control represents the mean of results obtained with four independent RNA preparations, mean ± S.E. All other points represent the mean of results obtained with three independent RNA preparations, mean ± S.E.
Zero time point represents the mean of six independent assays, mean ± S.E. Each other point represents the mean of triplicate independent assays, mean ± S.E. Methods were as described in the legend to Fig. 1. Control untreated cultures (O); cultures treated with 100 ng/ml insulin (●).

Fig. 2. Time course for effect of insulin on albumin mRNA. Zero time point represents the mean of six independent assays, mean ± S.E. Each other point represents the mean of triplicate independent assays, mean ± S.E. Methods were as described in the legend to Fig. 1. Control untreated cultures (O); cultures treated with 100 ng/ml insulin (●).

FIG. 3. a, Northern blot of cytoplasmic RNA from control and insulin-treated cultures. Cells were treated for 24 h with varying concentrations of insulin. Each lane contained 10 μg of cytoplasmic RNA. The filter was hybridized with 32P-labeled pRSA13 DNA probe. Lanes 1 and 2, control untreated cultures; lane 3, +100 ng/ml insulin; lane 4, +10 ng/ml insulin; lane 5, +1 ng/ml insulin; lane 6, +0.1 ng/ml insulin; lane 7, +0.03 ng/ml insulin; lane 8, +0.01 ng/ml insulin; lane 9, +0.003 ng/ml insulin; lane 10, +0.001 ng/ml insulin. Positions of 18 S and 28 S ribosomal RNAs are indicated on the left. b, same experiment as in a, probed with 32P-labeled α-tubulin cDNA.

FIG. 4. Northern blot of poly(A) + cytoplasmic RNA from control and insulin-treated cultures. Cells were treated for 24 h with varying concentrations of insulin. Cytoplasmic RNA was prepared and the poly(A) + fraction was purified as described under "Materials and Methods." Each lane contained 1 μg of RNA. Lane 1, control untreated cultures; lane 2, +1 ng/ml insulin; lane 3, +10 ng/ml insulin; lane 4, +100 ng/ml insulin. The filter was hybridized with 33P-labeled pRSA13 DNA probe. Positions of 18 S and 28 S ribosomal RNAs are shown at the left.
were 6.86 under "Materials and Methods." Each bar indicating range for duplicates.

and the cells in C and D were completely confluent. Cell densities at levels were then determined by dot blot hybridization as described additional then added to each flask, and the incubation was continued for an additional 3 days. At this time, visual inspection indicated that the cells in A were subconfluent, the cells in B were nearly confluent, and the cells in C and D were completely confluent. Cell densities at this time, as determined by counting cells in parallel control cultures, were 6.66 × 10^5 cells/flask (set A), 1.19 × 10^6/flask (set B), 1.69 × 10^5 cells/flask (set C), and 1.72 × 10^5 cells/flask (set D). Insulin was added to half of the cultures (solid bars) and incubation was continued for 24 h. The other half of the cultures (open bars) were control cultures incubated in the absence of insulin for 24 h. Albumin mRNA levels were then determined by dot blot hybridization as described under "Materials and Methods." Each bar represents the results obtained with RNA from four independent cultures, mean ± S.E. except bar C, control, which is the average of duplicates with error bar indicating range for duplicates.

was changed at the time of insulin addition (bars C and D).

The results presented in Fig. 6 indicated that the effect of insulin on albumin mRNA was strongly dependent on the nutritional status of the cells. To determine what component of the medium might modulate the insulin response, we added essential amino acids, nonessential amino acids, or vitamins along with the insulin. The results (Fig. 7) indicated that the essential amino acids in the medium were primarily responsible for counteracting the insulin response.

To determine whether the negative effect of insulin on albumin mRNA was caused by a decrease in transcription, the effect of insulin on albumin gene transcription was examined in nuclear transcription elongation assays. The results (Fig. 8) indicated that treatment of the H4 cells with insulin for 24 h caused a decrease in transcription of the albumin gene. The average magnitude of this decrease in three independent experiments performed on different days was 4.7-fold. This effect was specific, in that insulin did not cause a significant decrease in transcription of the a-tubulin gene, which was used as an internal control (Fig. 8). These data indicate that the suppression by insulin of albumin mRNA levels in the H4 cells is caused, in part, by a negative effect on transcription of the albumin gene and, in part, by a post-transcriptional effect on albumin mRNA.

DISCUSSION

Treatment of the H4 rat hepatoma cells with insulin results in a dramatic decrease in albumin mRNA. This effect is

Fig. 5. Effect of insulin and varying cell density on albumin mRNA levels. H4 cells were plated at a density of 0.5 × 10^6 (A), 1.0 × 10^6 (B), 2.0 × 10^6 (C), or 4.0 × 10^6/75-cm² tissue culture flask in MEM plus 10% serum. After 3 days at 37°C, the medium was aspirated and the cells were washed once with serum-free MEM. Serum-free F-12/Dulbecco's modified Eagle's medium (10 ml) was then added to each flask, and the incubation was continued for an additional 3 days. The medium was then changed to fresh serum-free medium on some cultures (bars C and D) but not on other cultures (bars A and B). Insulin was then added to half of the cultures (bars B and D) and incubation was continued for 24 h. Albumin mRNA levels were then determined by dot blot hybridization as described under "Materials and Methods." Each bar represents the results obtained with RNA from four independent cultures, mean ± S.E. except bar C, control, which is the average of duplicates with error bar indicating range for duplicates.

Fig. 6. Effect of insulin and medium change on albumin mRNA levels. H4 cells were incubated in serum-free medium for 3 days. The medium was then changed to fresh serum-free medium on some cultures (bars C and D) but not on other cultures (bars A and B). Insulin was then added to half of the cultures (bars B and D) and incubation was continued for 24 h. Albumin mRNA levels were then determined by dot blot hybridization as described under "Materials and Methods." Each bar represents the results obtained with RNA from four independent cultures, mean ± S.E. A, no medium change, no insulin; B, no medium change, +100 ng/ml insulin; C, medium change, no insulin; D, medium change, +109 ng/ml insulin.

Fig. 7. Effect of insulin and nutritional manipulations on albumin mRNA. Cells were treated as indicated for 24 h, and albumin mRNA levels were determined by dot blot analysis as described under "Materials and Methods." Essential amino acids and vitamins were added at the concentrations that are present in MEM. Nonessential amino acids were added at a concentration of 0.1 mM. Each bar represents the average of duplicate determinations. A, control untreated cultures; B, +100 ng/ml insulin; C, +nonessential amino acids; D, +100 ng/ml insulin + nonessential amino acids; E, +essential amino acids; F, +100 ng/ml insulin + essential amino acids; G, +essential amino acids + nonessential amino acids; H, +100 ng/ml insulin + essential amino acids + nonessential amino acids; I, +vitamins; J, +100 ng/ml insulin + vitamins.
observed at very low doses of insulin, consistent with insulin exerting the effect by interacting with high affinity receptors. Insulin has been shown previously to lower mRNA levels and inhibit transcription of the phosphoenolpyruvate carboxykinase gene in the H4 cells (17-19). The ED<sub>50</sub> for the suppressive effect of insulin on albumin mRNA reported here is 2 pm, which is in good agreement with the ED<sub>50</sub> of 2-5 pm reported previously for suppression by insulin of transcription of the phosphoenolpyruvate carboxykinase gene in the same cells (19). Insulin does not decrease α-tubulin mRNA in the H4 cells, providing further evidence for specificity of the effect on albumin mRNA.

Nuclear transcription assays revealed that insulin decreased transcription of the albumin gene in the H4 cells. However, the 4.7-fold decrease in transcription cannot account completely for the 57-fold reduction in cytoplasmic albumin mRNA. It appears, therefore, that the suppression by insulin of cytoplasmic mRNA is also due in part to post-transcriptional effect(s). Post-transcriptional regulation of albumin mRNA has been described in two other systems. Albumin mRNA decreases rapidly in primary cultures of rat hepatocytes cultured in medium with serum, but is maintained at high levels in hepatocytes cultured in hormone-supplemented serum-free medium (35). The maintenance of high levels of albumin mRNA in serum-free medium appears to be caused by an increase in albumin mRNA stability (35).

In cultured *Xenopus* hepatocytes, estrogen causes a sharp reduction of albumin synthesis and cytoplasmic albumin mRNA (36, 37). This effect appears to involve destabilization of albumin mRNA (36). Very little is known at present regarding molecular mechanisms for regulation of the stability of albumin or other mRNAs.

The effect of insulin on albumin mRNA levels was strongly dependent on the nutritional status of the cells, and particularly, the availability of essential amino acids. Nutrition also strongly affects albumin biosynthesis in vivo. Deprivation of protein from the diet leads to a decrease in production of insulin and other secretory proteins by the liver in rats (38) and humans (39). Studies on albumin mRNA levels in rat liver have indicated that a short term fast causes a 40% decrease in albumin mRNA/unit of liver weight and a redistribution of the albumin mRNA from membrane-bound polysomes to free polysomes and the post-ribosomal supernatant fraction (40). An analysis of albumin synthesis by cultured mouse hepatoma cells has indicated that limitation for essential amino acids causes a 70% decrease in albumin synthesis, resulting from a reduced number of functional albumin messages/cell (41). These findings are consistent with the possibility that amino acid limitation could cause a redistribution of albumin mRNA from a more stable to a less stable fraction. The molecular mechanism by which insulin destabilizes albumin mRNA in the H4 hepatoma cells, and the relationship between this effect and limitation for essential amino acids, remains to be elucidated.

Previous results obtained with diabetic rats have suggested that insulin is a positive regulator of albumin biosynthesis and albumin mRNA production (11, 12). However, diabetes is a complicated disease involving derangement of a variety of physiological processes and the positive effect of insulin on albumin biosynthesis in diabetic animals may not be caused by direct action of insulin. Insulin does not increase the levels of albumin mRNA in normal rats, whereas it does increase the level of mRNA for malic enzyme (14, 42). The kinetics for induction of the two mRNAs in diabetic animals are also different, suggesting a different mechanism for the regulation of albumin mRNA and malic enzyme mRNA (14, 42). Drake et al. (14, 42) have speculated that the positive effect of insulin on albumin mRNA in diabetic rats represents a general influence of insulin on RNA transcription, whereas the positive effect on malic enzyme mRNA is a specific inductive effect. *In vitro* experiments performed with cultured chick embryo hepatocytes have also suggested that insulin is a positive regulator of albumin mRNA production (13). However, these experiments were performed on cells in which the level of albumin mRNA was decreasing rapidly with time in culture. The effect of insulin was to partially reverse this decrease, thus a direct inductive effect of insulin was not demonstrated in this case either.

It is possible that insulin may be a positive regulator of albumin biosynthesis and that this effect is reversed in the H4 hepatoma cells. One other example of reversal of a hormonal effect in a cultured cell line has been reported recently. Dexmethasone is a negative regulator of α-fetoprotein mRNA levels and biosynthesis in neonatal rat liver and in the 777 rat hepatoma cell line (43, 44). In contrast, dexmethasone increases α-fetoprotein gene transcription and mRNA levels in the McA-RH8994 rat hepatoma cell line (44). There-
fore, positive as well as negative hormone-responsive regulation may occur for a single gene depending on the cell. This suggests that other factors, which have yet to be identified, also participate in the hormone responsiveness of the gene. The H4 hepatoma cells exhibit a variety of physiological responses to insulin and have been used widely as a cell culture model for studies of insulin action (16-19, 24-28). It would therefore be interesting if one normal physiological action of insulin were specifically reversed in these cells.

In a previous study of α-fetoprotein and albumin secretion by the H4 cells, insulin was reported to have a negligible effect on albumin accumulation in the growth medium, although insulin appeared to cause a small decrease in albumin secretion during a time period in which the cells were sensitive to insulin as determined by a positive effect on DNA synthesis (45). Effects of insulin on albumin gene transcription, mRNA levels, or rate of biosynthesis were not reported in this study. In addition, this study was performed with subconfluent cells, and the protocol involved frequent medium changes, both of which conditions greatly minimize the insulin effect on albumin mRNA (see Figs. 5 and 6). More recently, Tsuchada et al. (46) have reported that insulin does not alter albumin biosynthesis in a variant of the H4 cell line selected for ability to grow in protein- and lipid-free synthetic medium. It is not known whether the variant cell line has high-affinity insulin receptors, and physiological responsiveness of the variant to insulin has not been documented.

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