Direct effect of insulin on the synthesis of specific plasma proteins: Biphasic response of hepatocytes cultured in serum- and hormone-free medium

(crossed immunoelectrophoresis/albumin/fibrinogen/transferrin/acute phase proteins)

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ABSTRACT Monolayers of chicken embryo hepatocytes, cultured in chemically defined medium, retain the ability to synthesize a wide spectrum of plasma proteins for several days in the absence of added hormones. Addition of insulin to the medium elicited a biphasic stimulation of plasma protein synthesis: a rapid response of the synthesis of a limited number of plasma proteins (e.g., albumin and α1-globulin "M"), then, after prolonged exposure to the hormone, the involvement of additional plasma proteins (e.g., fibrinogen and lipoproteins). Synthesis of transferrin and a few other plasma proteins was not affected by the presence of insulin. The degree of stimulation for the most responsive plasma proteins ranged between 2- to 4-fold during the early phase and 10- and even 30-fold during the late phase of the cells' response to insulin. Stimulated synthesis in the early phase was detected within 1 hr and was rapidly reversible. Plasma protein synthesis in culture was sensitive to concentrations of insulin below 0.35 mM, well within the physiological range. The delayed response was elicited only at higher hormone levels. Parallels between the control of synthesis of plasma proteins in this system and that observed in diabetic animals suggest that the embryonic chicken hepatocytes may be a useful model for studying liver function in diabetes as well as insulin action in general.

The anabolic effect of insulin on carbohydrate metabolism in the liver is well documented (1). However, the role of insulin in regulating the hepatic function of plasma protein synthesis has not been clearly defined. In the early 1960s, experiments with perfused livers of diabetic rats showed a decrease in total plasma protein synthesis (2) as well as reduced synthesis of albumin (3) relative to that in normal rat liver. More recently, it has been reported that the lowered synthesis of hepatic secretory proteins by the diabetic rat can be restored to normal by injection of insulin (4).

To investigate the effect of insulin on plasma protein synthesis without the influence of other body organs, a number of rat liver-derived systems have been used, including isolated perfused liver (5) and dispersed cell suspensions (6, 7). These studies reported modest stimulation of albumin synthesis (less than 2-fold) with little, if any, effect on fibrinogen synthesis. Long-term responses to the hormone were not rigorously examined, due to the limited viability of the systems employed. Clear interpretation of the effect of insulin was further complicated by the simultaneous presence of serum (6, 7) or mixtures of hormones (5, 6). Some of these agents have been shown individually to stimulate the synthesis of specific plasma proteins in culture (8-10).

Our study was undertaken to examine the direct action of insulin alone on the hepatocellular synthesis of a wide range of plasma proteins. It has been made possible by development of a hepatocyte monolayer culture derived from chicken embryos, which can sustain synthesis of a broad spectrum of plasma proteins in medium free of serum, hormone, or other macromolecular supplement (9, 10). In this system, the rate of plasma protein synthesis is evaluated by measuring, with electroimmunoassays (11), the rate at which the secreted plasma proteins accumulate in the medium (12).

We report here that the plasma proteins can be separated, on the basis of their response to insulin, into those that are stimulated immediately, those that are stimulated only after prolonged exposure to the hormone, and those that are not affected at all.

MATERIALS AND METHODS

The preparation of hepatocyte suspensions from 16-day-old chicken embryos by perfusion of the liver, mechanical disruption, and treatment with purified dissociating enzymes was performed essentially as described (8, 12). Modified Ham's F-12 medium (13) was used without insulin or serum supplementation so that the cells were washed, plated, and maintained in chemically defined hormone-free medium. The hepatocyte suspensions were plated in 2-ml volumes in 35-mm-diameter plastic culture dishes (Falcon), which received no special treatment prior to use. The resultant monolayers contained approximately 10^6 cells, equivalent to 1 mg of cell protein per dish (12); where noted, larger dishes were used. Culture medium was replaced with an equal volume of fresh medium every 24 hr unless otherwise indicated. Heparin sodium salt was included, at a concentration of 15 µg/ml, in medium intended for fibrinogen assay (8). Insulin (bovine pancreas, crystalline, Sigma, 24.3 units/mg) was routinely used. Glucagon-free insulin (porcine, crystalline, 27.5 units/mg), a gift from Lilly Research Laboratories, gave similar results. Stock solutions of insulin (1 mg/ml) were prepared and stored in 0.01 M HCl. Bovine serum albumin (fatty acid-free) was from Pentex (Miles).

Plasma protein synthesis was determined by measuring secreted plasma proteins in 3-µl samples of unconcentrated culture medium, using electroimmunoassays with monospecific and polyspecific antisera as described (10-12, 14). Antiserum to chicken α1-acid glycoprotein was prepared as described elsewhere (10). Rabbit anti-conalbumin, which recognizes chicken transferrin, was a generous gift from R. D. Palmer of the University of Washington, Seattle, WA. Crossed immunoelectrophoresis was performed as described (15) on samples of culture medium that were concentrated by ultrafiltration with supported YM-10 membranes (Amicon). Intracellular albumin

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levels were determined in liver cell homogenates by electroimmunoassay as described (12).

Total protein synthesis was determined by the following protocol: Beginning at 3 hr after plating, the cells were exposed to insulin (35 nM), for various lengths of time. Before the end of each interval, they received 1-[35S]leucine (Schwarz/Mann, specific activity 6 Ci/mmol, 1 Ci = 3.7 × 10^10 becquerels) at a concentration of 5 μCi/ml for 15 min, a period brief enough to ensure that most of the labeled plasma proteins still remained within the cells (12). In every case, culture medium was changed 2 hr prior to pulse labeling. Incorporation of label into cellular material precipitable by hot trichloroacetic acid was determined as described (12).

DNA synthesis was estimated by autoradiographic analysis of [3H]thymidine incorporation. Three hours after plating, one set of culture dishes was incubated with [methyl-3H]thymidine (Schwarz/Mann, specific activity 1.9 Ci/mmol) at 0.5 μCi/ml in fresh medium (controls) or fresh medium plus 35 nM insulin for a period of 24 hr. A second set of dishes received fresh medium with or without insulin for two consecutive 24-hr intervals, radiolabel being included only in the second interval. After the labeling period, the cells were incubated for an additional 18 hr in fresh medium supplemented with 1000-fold excess unlabeled thymidine, then fixed with 2.5% (vol/vol) glutaraldehyde; the autoradiographs were prepared and evaluated as described (10).

Data given in this report are representative of a minimum of three separate experiments.

RESULTS

Primary cultures of chicken embryo hepatocytes synthesize a broad spectrum of plasma proteins for several days when maintained, from the onset of culture, in a chemically defined medium without serum, stabilizing protein, or hormonal supplement (Fig. 1 A and C; Table 1). The plasma proteins identified in Fig. 1 include albumin, fibrinogen, transferrin, lipoproteins, α-antitrypsin, and α1-globulin "M." This latter protein is an adult-type plasma protein (15) that is also a major acute phase reactant in the chicken (unpublished results). Several plasma proteins not recognized by the antibody chosen for the illustration (Fig. 1) were measured in the culture medium only by electroimmunoassay with specific antisera: α2-macroglobulin, α1-acid glycoprotein, plasminogen, and a prealbumin, designated "C" (15), the function of which is yet unknown.

Addition of insulin to the culture medium altered the pattern of plasma protein synthesis, and, as the length of exposure to the hormone was extended, the pattern changed qualitatively and quantitatively (Fig. 1). On the first day of exposure, insulin

![FIG. 1. Crossed immunoelectrophoresis of secreted plasma proteins synthesized in the presence and absence of 35 nM insulin. Twenty-four hours after plating, the cells were given fresh heparin-containing medium, with or without insulin. The medium was collected 24 hr later—i.e., at the end of the second day of culture—and the cells were reexposed to fresh medium as before, with or without insulin. After an additional 24-h incubation—i.e., at the end of the third day of culture—the medium was again collected. Medium samples, concentrated 400-fold, were applied (5 μl) in the appropriate well in the lower left corner of each panel. Electrophoresis in the first dimension was performed from left to right and in the second dimension from bottom to top. The second-dimension gel (antibody-containing) contained immunoglobulin fractions prepared from the antisera by ammonium sulfate precipitation: 27.8 mg, equivalent to 32.6% antiadult chicken serum (i.e., 46 μl per cm^2) and 1.92 mg, equivalent to 2.2% anti-chicken fibrinogen (i.e., 3 μl per cm^2). Immunoplates were stained with Coomassie blue. In this assay, the amount of each plasma protein is reflected by the intensity and area of its respective peak (15). In order to match the corresponding peaks in A-D, the samples of culture medium were compared by tandem crossed immunoelectrophoresis (12). Several peaks have been identified with the use of specific antisera or purified antigens and are numbered according to refs. 8 and 12: peak 4, albumin; peak 6, α1-globulin "M"; peak 13, prealbumin; peak 17, α-antitrypsin; peak 19, transferrin; peak 21, fibrinogen. Unlike the anti-chicken serum used in the above references, this antiserum did not recognize prealbumin "C." Plasma protein synthesis on the first day of exposure is shown in controls (A) and in the presence of insulin (B); synthesis on the second day of exposure is shown in controls (C) and in the presence of insulin (D).](http://www.pnas.org/content/78/11/6973)

Table 1. Plasma protein synthesis in hormone-free medium

<table>
<thead>
<tr>
<th>Day</th>
<th>Fibrinogen</th>
<th>α1-Globulin &quot;M&quot;</th>
<th>Transferrin</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.6 ± 0.2</td>
<td>0.30 ± 0</td>
<td>16 ± 1</td>
<td>22.7 ± 0.9</td>
</tr>
<tr>
<td>1</td>
<td>1.5 ± 0.1</td>
<td>0.89 ± 0.04</td>
<td>26 ± 2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>1.4 ± 0.1</td>
<td>1.07 ± 0.06</td>
<td>24 ± 2</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>1.5 ± 0</td>
<td>1.21 ± 0.09</td>
<td>19 ± 2</td>
<td>0.20 ± 0.02</td>
</tr>
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Cells were plated and maintained in 55-mm-diameter dishes in 5 ml of medium without supplement. Secreted plasma proteins were determined individually with specific antisera by electroimmunoassay of medium samples taken every 24 hr, immediately before fresh medium was given. Samples for determination of fibrinogen were taken from two separate sets of dishes that had received fresh medium containing heparin, beginning either at 3 hr after plating (for day 1 synthesis) or at 24 hr (for days 2, 3, and 4). Values represent the mean ± SD of triplicate dishes.
electroimmunoassay with specific antibodies (14). In contrast, insulin had no effect, during either the early or the late phase of the cells' response, on the synthesis of certain plasma proteins, including α₁-acid glycoprotein (not shown), transferrin, and proteins 1 and 12.

The effects of varied concentrations of insulin on the synthesis of albumin and fibrinogen were examined on the first and second day of hormone exposure (Fig. 2). During the early interval (Fig. 2A), significant stimulation of albumin synthesis was first detectable at 0.35 nM and reached a maximal level at 3.5 nM insulin. For half-maximal stimulation, a concentration of 1 nM was required. Fibrinogen synthesis was not affected over the entire range of hormone concentrations (up to 350 nM). During the late interval (Fig. 2B), insulin increased fibrinogen synthesis, but only at relatively higher doses of the hormone. Maximal synthesis of both fibrinogen and albumin required 35 nM insulin at this time. Although this concentration is 10 times higher than that needed for maximal response of albumin synthesis in the earlier interval, it does not reflect a decreased sensitivity of the cells toward the hormone. Insulin (3.5 nM) stimulated albumin synthesis about 4-fold during the late phase, an enhancement equivalent to that produced by this concentration of insulin during the early phase (Fig. 2 A and B).

It is difficult to assess the minimal insulin concentration that will elicit a response in culture because liver cells degrade insulin (16) and the hormone adheres to glass as well as plastic surfaces (17). The effective concentration of insulin may therefore be considerably lower than the concentrations plotted in Fig. 2. To reduce the nonspecific binding, we included bovine serum albumin (0.1 mg/ml) in a side experiment otherwise identical to that described in Fig. 2. Bovine serum albumin itself had no discernible effect on plasma protein synthesis, but its presence increased the cells' apparent sensitivity to insulin by an order of magnitude such that maximal responsiveness of albumin synthesis was observed in the early phase at 0.35 nM and in the later phase at 3.5 nM (data not shown).

In the absence of insulin, several major plasma proteins were synthesized at an undiminished rate over 4 days (Table 1). However, albumin synthesis, which initially accounted for 50% of plasma protein synthesis (12), decreased rapidly to very low levels by the third day of culture (see also Fig. 3, curve E). This declining synthesis of albumin, unlike that associated with loss of viability, occurred in cells that had a fairly constant rate of total protein synthesis—e.g., 15,500 ± 900, 17,300 ± 1600, and 15,100 ± 1000 cpm per dish, at 5, 29, and 77 hr of culture, respectively.

Whereas addition of insulin (35 nM) stimulated the rate of albumin synthesis, it did not initially prevent the overall decline of albumin synthesis with time (Fig. 3). Prolonged exposure, however, reversed the downward trend by dramatically increasing albumin synthesis. Biphasic kinetics of stimulation characterized the response of albumin synthesis to insulin regardless of whether exposure was begun at 0, 9, 23, or 47 hr after plating. In each case, the rate of albumin synthesis, although initially stimulated about 2-fold by insulin, declined during the

**Fig. 2.** Effect of increasing concentrations of insulin on the synthesis of albumin and fibrinogen. Twenty-four hours after plating, the cells were first exposed to various concentrations of insulin, as indicated on the abscissa, in fresh medium containing heparin. After another 24 hr, samples were taken and incubation in fresh medium, with hormone as before, was continued for a second 24-hr interval, at the end of which samples were again taken. Secreted plasma proteins were determined by electroimmunoassay and are expressed as the mean of duplicate dishes in μg per ml of medium. (A) First 24-hr exposure to hormone; (B) second 24-hr exposure to hormone.

**Fig. 3.** Response of albumin synthesis to insulin (35 nM) added at various times during culture. Cells were plated and maintained in medium containing heparin, which was used throughout this experiment. Insulin was added to the medium, as indicated by arrows, either (curve A) at the time of plating—i.e., 0 hr—or at the following times after plating: curve B, 9 hr; curve C, 23 hr; curve D, 47 hr. Curve E received no hormone. Once insulin had been introduced, its presence was maintained in later medium changes at 23, 47, 71, and 95 hr. Secreted albumin was determined by electroimmunoassay; cumulative total values, expressed as μg per ml, are plotted.
long lag period before undergoing recovery. The recovered rate of albumin synthesis, in each case, was about 8 μg/ml per day, representing as much as a 30-fold enhancement over the control

Because many cell types respond to insulin by an increased rate of growth (18, 19), we investigated the effect of the hormone on DNA and total protein synthesis. Total protein synthesis was increased 1.3- to 1.9- and 2.4-fold in cells exposed to insulin (35 nM) for 2, 26, and 74 hr, respectively. DNA synthesis was monitored by autoradiography of cultures pulsed with [3H]thymidine during either 24- or 48-hr exposure to insulin (see Materials and Methods). In either case, the number of labeled hepatocyte nuclei did not differ from the control level, which was less than 5% labeling. These findings support the conclusion that the large enhancement of plasma protein synthesis occurring in the presence of insulin is not due to an increased number of hepatocytes. Nor is it due to nonuniform responsiveness of the hepatocytes to the hormone; we have shown that changes in albumin synthesis with length of exposure to insulin (as in Fig. 3, curve A) are reflected first in a corresponding decrease and then an increase of specific albumin immunofluorescence, which was characteristic of all the parenchymal cells in the monolayers examined (14).

To learn more about the initial response of the cells to insulin, the early kinetics of albumin stimulation were examined (Fig. 4A). A 2-fold increase of secreted albumin was detected as early as 1.5 hr after addition of the hormone. Thirty minutes after hormone exposure, intracellular albumin levels were found to be 2.9 ± 0.2 μg per control dish and 3.3 ± 0.2 μg per insulin-treated dish in an experiment similar to that described in Fig. 4A. This indicates that de novo synthesis of the protein, and not discharge of intracellular pools, accounts for the rapid increase of the rate of secretion. Persistence of stimulation required the continuous presence of insulin. Within 3 hr after insulin-containing medium had been replaced by fresh hormone-free medium, albumin secretion returned to the control rate (Fig. 4B).

**DISCUSSION**

A direct effect of insulin on production of hepatocellular plasma proteins has been evaluated in primary monolayer cultures from chicken embryo liver. It has been possible to test the effect of insulin alone in this system, because the cells do not require serum, hormones, or other macromolecular medium supplements for the synthesis of a variety of plasma proteins.

Exposure of these cells to insulin stimulated the synthesis of several plasma proteins. Moreover, the specificity of the insulin effect changed with the length of exposure and hormone concentration. This complex response to insulin can be resolved into two components: the initial response is detectable within 1 hr and involves a limited number of plasma proteins. The later response occurs with a lag of about a day, involves additional plasma proteins, and is elicited only at insulin concentrations 10 times higher than are required to produce the first effect.

The apparent concentration of insulin required for maximal stimulation in culture was 3.5 nM in the first phase and 35 nM in the later phase of the response. Upon reducing nonspecific binding of insulin by addition of bovine serum albumin, we were able to stimulate plasma protein synthesis with hormone concentrations below 0.35 nM, well within the physiological range; circulating levels of insulin in 16-day-old chicken embryos and in chickens are 0.1 and 0.3 nM, respectively (20). A role for insulin in the modulation of plasma protein synthesis in vivo may be indicated by the fact that the liver experiences fluctuations in insulin levels (21) over the same general range as has been shown to produce a response of plasma protein synthesis in culture.

We know of no other report describing the isolated effect of insulin on the synthesis of specific plasma proteins and examining both early and delayed effects of the hormone directly at the level of hepatocellular plasma protein biosynthesis. In these experiments, evaluation of the effect of insulin was extended to the entire spectrum of secreted plasma proteins. Plasma protein accumulation in the medium was measured by electrophoresis, an approach that gives a direct measure of the absolute rate of plasma protein synthesis in this system (11, 12).

The assay is not influenced by changes in precursor pools, which may be the case when determinations are based on incorporation of labeled amino acids into protein. This is a particular advantage in these studies, because insulin is known to stimulate amino acid uptake (22) and inhibit protein degradation (23), potentially affecting cellular amino acid pool sizes.

Three classes of plasma proteins were observed in terms of insulin’s effect on their synthesis in culture: (i) very responsive proteins (e.g., albumin, α1-globulin M, prealbumin C, α-antitrypsin, and α2-macroglobulin); (ii) proteins that are affected only in the late phase of the response (e.g., fibrinogen, plasminogen, and the lipoproteins); and (iii) proteins whose synthesis is not affected at all (e.g., transferrin and α2-acid glycoprotein). In the only other published study with insulin as the sole hormone additive, no increase was observed, during a 12-hr perfusion, in the synthesis by isolated rat liver of the plasma proteins α1-acid glycoprotein, albumin, and fibrinogen (24).

In our system, fibrinogen synthesis did not respond to insulin until
the second day of exposure; hence during the 12-hr perfusions the hormone would not be expected to enhance synthesis of this protein. It is not clear, however, why insulin exerted no effect on albumin synthesis in this perfused rat liver system.

The mechanisms underlying the biphasic effect of insulin on plasma protein synthesis remain to be determined. The rapid stimulation and dissipation of the immediate response to insulin are consistent with the hypothesis that insulin initially acts on plasma protein synthesis via a posttranscriptional mechanism. Indeed, when insulin was added to the cells in the presence of the transcriptional inhibitor actinomycin D, the immediate stimulation of albumin synthesis was not blocked (unpublished). In contrast, the stimulation of albumin synthesis in the late phase of the response was largely abolished in the presence of the inhibitor, reflecting the probable involvement of an additional, transcriptional component after prolonged exposure.

The increase by insulin of albumin messenger RNA levels in livers of diabetic rats, observed by Peavy et al. (4), supports the notion that the hormone can influence a pretranslational step in the biosynthesis of albumin.

Despite the fact that most plasma proteins are synthesized by these hepatocyte cultures at steady rates for several days in the absence of added hormones, there is a dramatic decline in the synthesis of albumin and, conversely, an induction of the synthesis of adult-type plasma proteins—e.g., the α1-globulin M. The reduction of albumin synthesis with time in culture is specific (Fig. 1 and Table 1), unlike that associated with loss of viability in some other systems (6, 7). This behavior of albumin synthesis in our cultures is unchanged by inclusion of serum in the medium (15) and appears to be related to a decline of albumin messenger RNA (unpublished data). Although a number of hormones have been shown to stimulate synthesis of specific plasma proteins in this culture system (8, 9), none can replace the requirement of the cells for insulin to restore albumin synthesis. Insulin is also required in primary frog hepatocyte cultures for continued albumin synthesis (25).

The behavior of plasma protein synthesis in our cultures has several parallels in vivo which suggest that the cultured embryonic chicken hepatocytes may provide a suitable model, relatively easy to manipulate, for studying liver function in diabetes. A reduction of plasma protein synthesis is seen both in the insulin-free cultures of chicken hepatocytes, relative to the rate observed under optimal culturing conditions, and in the liver of the alloxaan-diabetic rat, relative to levels of synthesis in the normal rat (4); in both systems, albumin synthesis is disproportionately decreased. In our hormone-free cultures, total protein synthesis is about 40% of that observed in the presence of insulin, whereas albumin synthesis drops in 3 days to 1%. Likewise, in the liver of a 72-hr diabetic rat, the rates of total protein synthesis and specific albumin synthesis were estimated to be reduced to 50% and 8%, respectively (4). Prolonged exposure to insulin, in these cultures and in the diabetic rat, markedly stimulates plasma protein synthesis, restoring albumin synthesis almost to control levels. Thus insulin, in addition to its well-known function in regulating hepatic carbohydrate metabolism, may play a major role in maintaining and regulating the synthesis of albumin and other plasma proteins in vivo.

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