Regulation of Malic Enzyme Synthesis by Insulin, Triiodothyronine, and Glucagon in Liver Cells in Culture*

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Cells isolated from the livers of 17- to 19-day-old chick embryos were maintained in a chemically defined culture medium. During 3 days in culture the activity of malic enzyme, a measure of its concentration, was stimulated 2-, 23-, or 77-fold by insulin, triiodothyronine, or insulin plus triiodothyronine, respectively. Glucagon blocked the stimulation caused by insulin plus triiodothyronine. Changes in the relative synthesis of immunologically isolated malic enzyme were similar in magnitude and direction to the changes in enzyme activity.

Degradation of malic enzyme was unaffected by the three hormones. Both soluble protein and malic enzyme were degraded with a $t_{1/2}$ of about 30 hours.

In cells preincubated for 2 days with insulin, synthesis of malic enzyme was stimulated 4.5-fold within 3 hours after adding triiodothyronine and reached an apparent new steady state after 24 to 30 hours. If the rate of enzyme synthesis was dependent on the concentration of cytoplasmic malic enzyme messenger RNA, then this messenger RNA appeared to have a $t_{1/2}$ of about 18 hours. Glucagon rapidly and specifically inhibited synthesis of malic enzyme in preincubated cells, suggesting an action at the level of translation or cytoplasmic messenger RNA processing.

Changes in fatty acid synthesis and the activity of malic enzyme which occur in vivo during starvation or starvation followed by refeeding can be mimicked quantitatively in liver cells cultured in a medium supplemented with horse and fetal calf serum. Thyroid hormone and one or more unknown serum factors stimulate and glucagon inhibits fatty acid synthesis and malic enzyme activity in these cells (1). We report here that malic enzyme is induced by thyroid hormone in liver cells incubated in a chemically defined medium. The induction is enhanced by insulin and inhibited by glucagon. The changes in enzyme concentration are due to changes in the relative synthesis of malic enzyme; degradation of the enzyme is unaffected by the hormones.

**EXPERIMENTAL PROCEDURE**

Preparation and Maintenance of Isolated Cells—Unincubated embryonated eggs from white Leghorn chickens were incubated in an electric forced-draft incubator at 37.5 ± 0.5°C and 60% relative humidity. Embryos (17 to 19 days of age) were killed by decapitation and liver cells isolated as previously described (1). The isolated cells were suspended in Waymouth Medium MD 705/1 (2) containing penicillin (60 μg/ml) and streptomycin (100 μg/ml). One milliliter of the suspension was added to the plates (3), were detached by gentle agitation with a flow of medium from a Pasteur pipette. The cells were then collected by centrifugation at 200 x g for 5 min. The supernatant (medium) was discarded and the cells washed once with Krebs-bicarbonate buffer. The washed cells were homogenized in 0.25 M sucrose/1 mM dithiothreitol/0.1 mM EDTA/5 mM Tris-HCl, pH 7.4, in a tight fitting homogenizer (Kontes Glass Co.). The cells from about three plates were homogenized in 1 ml. If only activity was to be measured, the homogenate was centrifuged at 200 x g for 5 min. The supernatant (medium) was discarded and the cells washed once with Krebs-bicarbonate buffer. The washed cells were homogenized in 0.25 M sucrose/1 mM dithiothreitol/0.1 mM EDTA/5 mM Tris-HCl, pH 7.4, in a tight fitting homogenizer (Kontes Glass Co.). The cells from about three plates were homogenized in 1 ml. If only activity was to be measured, the homogenate was centrifuged at 48,000 x g for 60 min. For immunoprecipitation studies, the homogenate was centrifuged at 130,000 x g for 60 min. Enzyme activity was assayed (4) in the supernatant fractions. Protein was measured by the method of Lowry et al. (5) with crystalline bovine albumin as standard.

Leucine Incorporation—After the experimental incubation period, the medium was removed and the attached cells gently washed with leucine-free Waymouth medium. Five milliliters of the leucine-free medium, modified to contain [3H]leucine (50 μCi/mM, 10 μCi/ml) were added and the plates incubated for 30 to 120 min. The cells were harvested and fractionated as described above. Protein was precipitated from portions of the total homogenate and the cytosol fraction with 10% trichloroacetic acid at 90°C (6, 7) and assayed for radioactivity.

Malic enzyme in the cytosol was further purified by adding MgCl2 to the cytosol fraction to a final concentration of 0.06 M and heating the samples to 58°C for 4 min (8). Malic enzyme was immunoprecipitated from the supernatant after removing the heat-precipitated protein by centrifugation. Little or no loss of enzyme activity occurred at the heat step. Malic enzyme (0.6 unit of endogenous plus carrier enzyme in a volume of 1 ml) was precipitated with 150 μl of antimalic enzyme rabbit serum (an amount sufficient to precipitate 1.2 units of enzyme). The reaction mixture contained 50 mM Tris-HCl/150 mM NaCl/10 mM DL-leucine/1% Triton X-100, pH 7.4, in a final volume of 1.65 ml. This
mixture was incubated at 37°C for 30 min and then overnight at 4°C. The precipitates were collected by centrifugation and washed twice with ice cold (150 mM NaCl/10 mM tris HCl/0.5% Triton X-100. The washed immunoprecipitates were dissociated in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol (100°C for 5 min) and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After electrophoresis, the gels were frozen and sliced. The slices were dissolved by heating overnight at 60°C in 0.5 ml of 30% H2O2. The precipitates were dissociated in 1% sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After electrophoresis, the gels were frozen and sliced. The slices were dissolved by heating overnight at 60°C in 0.5 ml of 30% H2O2. The precipitates were collected by centrifugation and washed twice with ice cold (150 mM NaCl/10 mM tris HCl/0.5% Triton X-100. The washed immunoprecipitates were dissociated in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol (100°C for 5 min) and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (8, 9).

More recently we have immunoprecipitated malic enzyme directly from the cytosol fraction eliminating the heat step. Instead, sodium dodecyl sulfate (0.2%) was added to the immunoprecipitation mixture to reduce nonspecific precipitation. Recovery of malic enzyme radioactivity was the same by both procedures.

Protein Degradation—After 2 days of culture, the medium was removed and replaced with 10 ml of Waymouth medium containing [3H]alanine (2 µM, 21 FCi/ml). The cells were incubated for 2 hours, after which the radioactive medium was removed and the cells gently washed with nonradioactive medium. After an additional 3 to 4 hours of incubation in Waymouth medium, the medium was changed again and one set of plates harvested for zero time values. Additional groups of incubation in Waymouth medium, the medium was changed again and one set of plates harvested for zero time values. Additional groups of incubation in Waymouth medium, the medium was changed again and one set of plates harvested for zero time values. Additional groups of incubation in Waymouth medium, the medium was changed again and one set of plates harvested for zero time values. Additional groups of incubation in Waymouth medium, the medium was changed again and one set of plates harvested for zero time values. 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Freshly isolated cells were incubated for 3 days with the additions shown in the table. Results are expressed as a percentage of the values for cells incubated with insulin plus triiodothyronine alone. Actual values for this group are indicated in parentheses ± S.E. Malic enzyme activity is expressed as milliunits/mg of total cellular protein; total protein as milligrams of total cellular protein; leucine incorporation into total cellular protein as disintegrations per min in total cellular protein × 10⁻⁷/hour/mg of total cellular protein; relative synthesis of malic enzyme as disintegrations per min in malic enzyme/100 dpm in total cellular protein; and relative synthesis of soluble protein as disintegrations per min in soluble protein/dpm in total cellular protein. All hormones were added at 1 μg/ml.

| Activity measured          | No. of experiments | No. of additions | Insulin | Triiodothyronine | Insulin + triiodothyronine | Insulin + triiodothyronine + glucagon |
|---------------------------|--------------------|------------------|---------|-----------------|---------------------------|--------------------------------------|
| Activity of malic enzyme  | 7-12               | 1.3 ± 0.3        | 2.5 ± 0.3 | 30 ± 6          | 100 (136 ± 12)            | 24 ± 0.3                             |
| Relative synthesis of malic enzyme | 3                 | 0.8 ± 0.2        | 1.1 ± 0.07 | 58 ± 5          | 100 (0.07 ± 0.34)         | 2.8 ± 0.8                            |
| Mass of total protein     | 9-13               | 71 ± 4           | 98 ± 4   | 64 ± 4          | 100 (2.37 ± 0.18)         | 89 ± 6                               |
| Incorporation of leucine  | 5                  | 51 ± 5           | 71 ± 5   | 94 ± 10         | 100 (3.98 ± 0.38)         | 98 ± 8                               |
| into total protein        |                    |                  |          |                 |                           |                                      |
| Mass of soluble protein   | 5-9                | 108 ± 8          | 125 ± 9  | 102 ± 5         | 100 (0.40 ± 0.03)         | 97 ± 8                               |
| Relative synthesis of     |                    |                  |          |                 |                           |                                      |
| soluble protein           | 1                  | 92               | 104      | 98              | 100 (0.51)                | 112                                  |

When expressed as a percentage of the synthesis at 24 hours (Fig. 3), the 3-hour value showed considerable variability. However, relative synthesis at 3 hours was 4.5 ± 0.6 (mean ± S.E.M., N = 5) times relative synthesis of the enzyme at zero time (p < 0.01). The increase caused by triiodothyronine was approximately linear for 24 hours (Fig. 3D), after which it appeared to reach a plateau (Fig. 4D). Twenty-four hours after adding triiodothyronine, relative synthesis of malic enzyme was 36 times that at zero time and 20 times that of the control cells (Fig. 3D). Incorporation of leucine into total protein was not significantly stimulated by triiodothyronine (Fig. 3B).

**DISCUSSION**

Glucagon, insulin, and thyroid hormone play important roles in regulating the synthesis of malic enzyme in liver cells in culture. How do these effects relate to the regulation of malic enzyme synthesis in vivo? In starved animals, malic enzyme synthesis is inhibited (8) and the plasma glucagon concentration is elevated (11-13). Hence, glucagon may be the signal in...
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FIG. 2. Effect of hormones on the degradation of soluble and malic enzyme protein in liver cells in culture. Degradation of soluble protein (A to E) and malic enzyme (F to J) is expressed as a percentage of the radioactivity in the respective proteins at the beginning of the degradation measurement. Symbols are the same as in Fig. 1. The original data were: malic enzyme, $7.1 \times 10^6$ dpm/2 hours/mg of soluble protein for cells preincubated with both high and low concentrations of triiodothyronine; soluble proteins, $1.9 \times 10^6$ and $2.5 \times 10^6$ dpm/2 hours/mg of soluble protein for cells preincubated with high and low triiodothyronine, respectively. Average results from two or three experiments are shown. Each experiment was performed in duplicate or triplicate.

FIG. 3. Time course for the increase in activity and relative synthesis of malic enzyme caused by triiodothyronine. After 2 days of incubation with insulin, the medium was changed to insulin (O) or insulin plus triiodothyronine (O). The cells were incubated with $[^{3}H]$leucine for $1/2$ or 1 hour, harvested, and assayed for total protein (A), leucine incorporation into total protein (B), malic enzyme activity (C), and relative synthesis of malic enzyme (D). Hormones were added at 1 ng/ml. Results are expressed as a percentage of the value for cells incubated for 24 hours in the presence of insulin plus triiodothyronine. Each point represents the mean ± S.E. of five experiments. Original values for the 24-hour incubated cells were: total cellular protein, 1.57 ± 0.17 mg/plate; leucine incorporation into total cellular protein, 2.7 ± 0.4 × 10^6 dpm/hour/mg of total cellular protein; malic enzyme activity, 48 ± 8 munitis/mg of total cellular protein; relative synthesis of malic enzyme, 0.26 ± 0.05 dpm/100 dpm in total cellular protein.

The stimulation of malic enzyme synthesis caused by triiodothyronine was blocked by α-amanitin (30), a specific inhibitor of eukaryotic RNA polymerase II, the enzyme respon-
then harvested and assayed for total cellular protein.

Nine. Then, the medium was changed again to contain either insulin or glucagon at 100 rig/ml. Results are expressed as a percentage of the value for the control cells at zero time. Each point represents the mean ± S.E. of five experiments. Original values for the control cells at zero time were: total cellular protein, 1.26 ± 0.14 mg/plate; leucine incorporation into soluble protein, 0.56 x 10^3 dpm/hour/mg of total cellular protein; malic enzyme activity, 61 ± 8

Fig. 4. Effect of glucagon on the activity and synthesis of malic enzyme in cells previously stimulated with triiodothyronine. After 2 days in the presence of insulin, the medium was changed and the cells incubated for 24 hours in medium containing insulin plus triiodothyronine. Then, the medium was again changed to contain either insulin plus triiodothyronine (A) or the same medium plus glucagon (B). At 0, 3, and 6 hours, the cells were incubated with [3H]leucine for 1 hour and then harvested and assayed for total cellular protein (A), leucine incorporation into soluble protein (B), malic enzyme activity (C), and relative synthesis of malic enzyme (D). Triiodothyronine and insulin were added at 1 µg/ml; glucagon at 100 ng/ml. Results are expressed as a percentage of the value for the control cells at zero time. Each point represents the mean ± S.E. of five experiments. Original values for the control cells at zero time were: total cellular protein, 1.26 ± 0.14 mg/plate; leucine incorporation into soluble protein, 0.56 x 10^3 dpm/hour/mg of total cellular protein; malic enzyme activity, 61 ± 8 milliunits/mg of total cellular protein; relative synthesis of malic enzyme, 1.1 ± 0.14 dpm/100 in soluble protein.

Table 1 shows the results of the experiment. The data indicates that the addition of glucagon to the medium caused a significant decrease in the activity of the malic enzyme. The relative synthesis of malic enzyme also decreased, suggesting that glucagon may inhibit the synthesis of malic enzyme messenger RNA.

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