Role of protein synthesis in the carbohydrate-induced changes in the activities of acetyl-CoA carboxylase and hydroxymethylglutaryl-CoA reductase in cultured rat hepatocytes

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Changes in the activities of acetyl-CoA carboxylase and HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase were studied in primary cultures of adult-rat hepatocytes after exposure of the cells to insulin and/or carbohydrates. To determine the contribution of protein synthesis to changes in enzyme activity, the relative rate of synthesis of each enzyme was measured and the amount of translatable mRNA coding for the enzymes was determined by translation in vitro and immunoprecipitation. Addition of insulin to the culture medium increased the activities of acetyl-CoA carboxylase and HMG-CoA reductase by approx. 4- and 3-fold respectively. Although similar increases in the relative rate of synthesis of each protein and template activity were noted, initial increases in the activity of each enzyme occurred before any changes in protein synthesis were observed, suggesting the involvement of post-translational modification of enzyme activity in addition to changes in protein synthesis. The addition of fructose to the culture medium, in the absence of insulin, increased the activity of the carboxylase and the reductase approx. 3-fold, similar to the effects of insulin. However, the effect of fructose was to increase the rate of synthesis and the amount of translatable mRNA coding for acetyl-CoA carboxylase, whereas the increase in the activity of HMG-CoA reductase was not accompanied by any changes in the rate of synthesis or template activity. The effects of fructose could not be mimicked by glucose unless insulin was also present in the culture medium. Similar to observations in vitro, the injection of insulin or the feeding of a high-fructose diet to rats made diabetic by the injection of streptozotocin produced an increase in the activities of acetyl-CoA carboxylase and HMG-CoA reductase, and only the increase in the activity of the carboxylase was accompanied by an increase in the amount of translatable mRNA coding for the enzyme. The results are discussed in terms of the effects of fructose on the synthesis of enzymes involved in lipogenesis.

Many of the lipogenic enzymes are regulated in parallel in their response to the hormonal and nutritional state of the animal (Gibson et al., 1972; Romso & Leveille, 1974; Volpe & Vagelos, 1976); however, the precise relationship between hormones and nutrients in this process is not entirely clear. Dietary fructose has been shown to be a more effective inducer of hepatic lipogenesis than glucose, despite the relatively weak effect of fructose on the secretion of insulin (Zakim et al., 1967). In addition, fructose, but not glucose, has been reported to stimulate lipogenesis in diabetic rats in the absence of insulin (Baker et al., 1952), and the activities of several lipogenic enzymes, including ATP citrate lyase (Kornacker & Lowenstein, 1965; Takeda et al., 1967), acetyl-CoA carboxylase (Takeda et al., 1967; Kumar, 1977), malic enzyme (Fukuda et al., 1983; Drake et al., 1984) and fatty acid synthetase (Volpe & Vagelos, 1974; Kumar, 1977), have been increased in livers of diabetic rats after feeding them with diets high in fructose content. These same enzyme activities

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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have been reported to increase in primary cultures of adult-rat hepatocytes after the addition of fructose to the culture medium in the absence of insulin (Spence & Pitot, 1982). The suggestion has been made that the mechanism by which dietary carbohydrate increases lipid synthesis is related to the breakdown of sugars and is not a direct effect of insulin or other hormones (Kornacker & Lowenstein, 1965). Although dietary fructose has been documented to produce an increase in circulating triacylglycerols as well as the hepatic synthesis of lipids (MacDonald, 1966; Nikkila, 1969; Rom sos & Leveille, 1974), the effect of carbohydrate feeding on the synthesis of cholesterol is more ambiguous and appears to be dependent on the species studied and the particular carbohydrate in question (Guynn et al., 1972; Rom sos & Leveille, 1974).

The regulation of lipogenesis and cholesterogenesis is complex, as indicated by the fact that the activities of the rate-limiting steps in each pathway, acetyl-CoA carboxylase (EC 6.4.1.2) and HMG-CoA reductase (EC 1.1.1.34) respectively, are controlled by both covalent modification and allosteric modulators (Kim, 1979; Gibson & Ingebritsen, 1978). Although such control mechanisms appear to be involved in short-term regulation of enzyme activity, the long-term control of lipogenesis appears to arise as the result of changes in the cellular content of particular enzymes (Guynn et al., 1972; Volpe & Vagelos, 1976). Thus, by examining the rate of synthesis of the enzymes involved as well as changes in the amount of translatable mRNA coding for those enzymes, one could differentiate the relative importance of short-term regulation from long-term regulation as mediated through changes in the rate of synthesis of the particular proteins. In this paper, we have used primary cultures of adult-rat hepatocytes to examine the insulin- and carbohydrate-induced changes in the activity of the rate-limiting steps of fatty acid biosynthesis and cholesterogenesis, namely acetyl-CoA carboxylase and HMG-CoA reductase. Using a translation assay in vitro to measure changes in the amount of translatable mRNA, we have examined the role that protein synthesis plays in the observed changes in activity.

**Experimental**

**Chemicals**

Components for tissue-culture medium were supplied by GIBCO (Grand Island, NY, U.S.A.); collagenase (type 1) was obtained from Cooper Biomedical (Freehold, NJ, U.S.A.) and insulin (bovine pancreas), avidin–agarose, Reactive Blue–agarose (type 3000), protein A–agarose and Triton WR-1339 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Agarose–hexane–HMG-CoA (type 5) was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.), and New England Nuclear (Boston, MA, U.S.A.) was the source for L-[4,5-3H]leucine, L-[14S]methionine, DL-3-hydroxy-3-methyl[3-14C]glutaryl-CoA and EN3HANCE. NaH14CO3 was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.) and gua-}

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Measurements of the relative rates of synthesis

The rate of synthesis of each enzyme was determined in the above homogenate by pulse-labelling of the cells with [3H]leucine 20 min before they were harvested, followed by the simultaneous immunoprecipitation of acetyl-CoA carboxylase and HMG-CoA reductase from the same sample. A 500 μl sample of the homogenate was incubated for 1 h at 37°C with the immunoglobulin fractions directed against each of the enzymes. Enough immunoglobulin was added to precipitate 10 μg of each protein. After the incubation, 25 μl of fixed Staphylococcus aureus cells (Immunoprecipitin; Bethesda Research Laboratories, Bethesda, MD, U.S.A.) was added and the sample incubated for an additional 30 min. The resulting immunoprecipitates were washed and separated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate; in order to prevent proteolysis, phenylmethanesulphonyl fluoride and tosyl-l-lysylchloromethane (2.5 mg/ml each) were included in all wash buffers, as described previously (Spence, 1983). The radiolabelled bands corresponding to the carboxylase and the reductase were localized by fluorography by using ENHANCE in accordance with the instructions of the manufacturer. The bands of interest were removed from the gel, dissolved, and counted for incorporation of [3H]leucine. Incorporation of radioactivity into total proteins was determined by precipitation of a sample of the original homogenate (25 μl) with trichloroacetic acid (Mans & Novelli, 1961). The relative rate of synthesis was determined for each protein and is expressed as c.p.m. incorporated into the carboxylase or the reductase/c.p.m. incorporated into total protein.

Translation of RNA in vitro

Plates of hepatocytes that were used for the isolation of RNA were harvested by removal of the culture medium and the addition of 1 ml of 4M guanidinium thiocyanate, and RNA was isolated as described by Spence & Koudelka (1984), except that total cellular RNA, not polyadenylated RNA, was used for the subsequent measurement of template activity. Translation of total cellular RNA in vitro was performed with a rabbit reticulocyte lysate translation assay and immunoprecipitation. The lysate was prepared and the reactions were performed as described previously (Spence, 1983) and contained up to 10 μg of RNA. Acetyl-CoA carboxylase and HMG-CoA reductase were isolated by immunoprecipitation from samples (20 μl) of the labelled translation products. A 2 μl sample of the translation reaction mixture was spotted on filter paper for the determination of radioactivity incorporated into total proteins as measured as

Tissue culture

Hepatocytes were isolated by collagenase perfusion and maintained on 100 mm-diameter culture dishes that had been coated with a solution of rat tail collagen as described previously (Spence et al., 1981; Spence & Koudelka, 1984). The culture medium used throughout these experiments was a modification of Leibovitz L-15, referred to as LHSA, in which the carbohydrate component of the medium, galactose, was omitted (Spence & Pitot, 1982). This carbohydrate-free LHSA was supplemented with newborn-calf serum (5%, v/v) and insulin (0.1 μM) only during the first 4 h of culture, after which time the serum and hormone were removed from the medium. The experiments were started after an additional 18 h incubation after the isolation of the cells by a change of medium with the indicated additions. Parallel sets of plates of cells were used for the determination of (1) enzyme activities and relative rates of synthesis, and (2) isolation of RNA.

Measurement of enzyme activities

Plates of cells that were used for the measurement of enzyme activities were harvested by adding 1 ml of buffer (50 mM-potassium phosphate, pH 7.2, 30 mM-EDTA, 100 mM-sucrose, 1 mM-dithiothreitol) to the plates of cells and scraping the cells from the dish with a rubber policeman. Homogenates of the cells were prepared with a Tekmar Tissuehomizer (Brinkmann Instruments) as described previously (Spence et al., 1981). The homogenates were then centrifuged at 100000 g for 1 h in a Beckman type 25 rotor. The resulting supernatant was used for the assay of acetyl-CoA carboxylase by the method of Craig et al. (1972). The microsomal pellet was solubilized and used for the assay of HMG-CoA reductase as described by Ness et al. (1979). Results are expressed as units/mg of DNA, where 1 unit of carboxylase activity represents 1 μmol of malonyl-CoA formed/min and 1 unit of reductase activity represents 1 μmol of mevalonate formed/min. The DNA content of the cells was determined as described by Bonney et al. (1974).
trichloroacetic acid-precipitable material as described above. Results are expressed as carboxylase or reductase template activity, which is defined as c.p.m. incorporated into the particular protein/c.p.m. incorporated into total protein.

The statistical evaluation throughout this paper was performed by Student's *t* test.

**Results and discussion**

*Translation of acetyl-CoA carboxylase and HMG-CoA reductase in vitro*

The amount of translatable mRNA coding for acetyl-CoA carboxylase and HMG-CoA reductase was determined by using a rabbit reticulocyte-lysate translation system and immunoprecipitation of the translation products. The translation of mRNA species coding for proteins of large *M*₅, such as acetyl-CoA carboxylase is particularly challenging. It was found that the reproducible determination of template activity was dependent on (1) the method of isolation of the RNA, (2) the purity of the antibody preparation used for immunoprecipitation, and (3) the inclusion of inhibitors of proteolysis in all buffers used to wash the immunoprecipitates. Several methods for the isolation of RNA were tried with limited success, apparently owing to degradation of the RNA. We found that the guanidinium thiocyanate procedure used for the isolation of RNA provided a fast and convenient method for the isolation of sufficient quantities of translatable RNA from a single plate of cultured cells, and the RNA was capable of directing the synthesis of large-*M*₅ proteins.

After translation of the RNA, acetyl-CoA carboxylase and HMG-CoA reductase were isolated from the translation products by immunoprecipitation with antiserum specific for the individual proteins. As presented in Fig. 1, acetyl-CoA carboxylase was translated as a single peptide of *M*₅, approx. 240000, similar to the value determined in vivo (Song & Kim, 1981; Goodson et al., 1984). HMG-CoA reductase was translated as a single peptide of *M*₅, approx. 90000, in agreement with estimates in vivo (Liscum et al., 1983) as well as observations of the protein synthesized by translation in vitro (Chin et al., 1982; Clarke et al., 1983). The identity of the proteins synthesized during translation in vitro was confirmed by using the highly purified carboxylase and reductase to compete for binding of the labelled proteins to the immunoglobulin (Fig. 1).

**Time course of changes in activity, rate of synthesis and template activity**

The effects of insulin on the activity, rate of synthesis and amount of translatable mRNA coding for acetyl-CoA carboxylase and HMG-CoA reductase were measured in primary cultures of adult-rat hepatocytes. When insulin was added to the culture medium, there was a rapid increase in the activities of both of these enzymes within 1–2 h, and maximal activities were noted at approx. 6 h after addition of the hormone to the culture medium (Fig. 2). For both the carboxylase and the reductase, changes in the activities of the enzymes were accompanied by an increase in the amount of translatable mRNA coding for the particular enzyme, as well as an increase in the rates of syn-
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Fig. 2. Effects of insulin on the induction of acetyl-CoA carboxylase and HMG-CoA reductase

Insulin (0.1 μM) was added to plates of cultured hepatocytes at 0 h and plates of cells were harvested at the indicated times. Measurements of enzyme activity (○), relative rate of synthesis (▲) and template activity (●) were performed for acetyl-CoA carboxylase (a) and HMG-CoA reductase (b). Each point represents the mean value ± S.D. from four determinations.

thesis of the enzymes. In each case, the increase in enzyme activity was observed before the increases in the rate of synthesis and the amount of translatable mRNA were apparent, suggesting that a portion of the increase, particularly that observed initially, was the result of post-translational regulation of enzyme activity. A similar observation of two different mechanisms for the increase in activity of another lipogenic enzyme, malic enzyme, has been reported (Thompson & Drake, 1982; Drake et al., 1980, 1983).

In addition to the hormonal effects on the activity of the two enzymes, the effects of fructose addition to the culture medium were examined. Fructose was added to the culture medium at zero time, in the absence of insulin (Fig. 3). The activities of both acetyl-CoA carboxylase and HMG-CoA reductase were increased to extents similar to those observed after addition of insulin to the culture medium. However, these fructose-induced changes in activity were delayed as compared with the effect of insulin; no detectable changes in the activity of either the carboxylase or the reductase were noted until 3 h after the addition of fructose. As was observed with insulin, the fructose-induced increase in the activity of acetyl-CoA carboxylase was accompanied by an increase in the rate of synthesis of the enzyme, along with an increase in the amount of translatable mRNA coding for the enzyme. Similar to the results that we obtained with the carboxylase, Katsurada et al. (1983) reported that the increase in the activity of malic enzyme after the feeding of fructose to rats was also the result of new protein synthesis, accompanied by an increase in template activity coding for the enzyme. The changes in enzyme and
Fructose was added to the culture medium of hepatocytes at a concentration of 25 mM, in the absence of insulin, and plates of cells were harvested at the indicated times. Measurements of enzyme activity (●), relative rate of synthesis (▲) and template activity (○) were performed for acetyl-CoA carboxylase (a) and HMG-CoA reductase (b). Each point represents the mean value ± S.D. from four determinations.

Fig. 3. Effects of fructose on the induction of acetyl-CoA carboxylase and HMG-CoA reductase

Fructose was added to the culture medium of hepatocytes at a concentration of 25 mM, in the absence of insulin, and plates of cells were harvested at the indicated times. Measurements of enzyme activity (●), relative rate of synthesis (▲) and template activity (○) were performed for acetyl-CoA carboxylase (a) and HMG-CoA reductase (b). Each point represents the mean value ± S.D. from four determinations.

In contrast with the carboxylase, the activity of HMG-CoA reductase was not accompanied by any changes in the rate of synthesis of the enzyme or changes in the amount of translatable mRNA coding for the enzyme, suggesting that the changes in activity may have arisen as the result of a post-translational mechanism, such as covalent modification of the enzyme or possible allosteric control.

Comparison of effects of fructose and glucose

A comparison was made of the abilities of fructose and glucose to increase enzyme activity (Table 1). Additions were made to the culture medium, and plates of cells were harvested after a further 6 h incubation. It was noted that the previously observed increases in the activity, rate of synthesis and template activity caused by fructose could not be mimicked by the addition of glucose to the culture medium. Glucose did, however, have effects similar to those of fructose when added to the culture medium in the presence of insulin. Fructose is more readily metabolized than glucose in the absence of insulin, which may be the
reason for the difference, in the light of the suggestion by Kornacker & Lowenstein (1965) that a metabolite arising from carbohydrate metabolism may play a critical role in the induction of lipogenic enzymes. The effects of fructose and insulin were additive with respect to changes in the activity of acetyl-CoA carboxylase as well as its rate of synthesis and template activity. However, the magnitude of the change in the activity of the enzyme under these conditions was much greater than the magnitude of the changes in the rate of synthesis and template activity, suggesting that the control of enzyme activity is not only a reflection of the synthesis of the protein. The effects of fructose and insulin were also additive with respect to changes in the activity of HMG-CoA reductase, but not for the rate of synthesis of the enzyme or the amount of translatable mRNA coding for the protein, suggesting that the response to the carbohydrate and to the hormone operate via different mechanisms.

The above results regarding the effects of glucose on the activity of acetyl-CoA carboxylase are seemingly in conflict with those presented by Katz & Ick (1981) and Gifihorn & Katz (1984), who found that glucose did have a direct effect on the activity of the enzyme. This difference between the observation in those reports and our results could be explained by differences in the culture conditions, in particular the presence of low concentrations of insulin in the culture medium in their experiments, which we have shown here could produce an effect with glucose.

**Effects of fructose on enzyme activity in streptozotocin-diabetic rats**

The above results suggest that the effect of insulin on the cultured hepatocytes was to increase the activity, rate of synthesis and template activity for both acetyl-CoA carboxylase and HMG-CoA reductase. Fructose could also increase the activity of both enzymes, but only the activity of acetyl-CoA carboxylase appears to arise as the result of new protein synthesis. To determine if the changes in enzyme activity arising from new protein synthesis observed in the cultured hepatocytes is indicative of effects that may occur in vivo, the effects of insulin and fructose were studied in rats made diabetic by injection of streptozotocin (Table 2). The activity of the carboxylase as well as that of the reductase were decreased by approx. 50% in the diabetic rats as compared with controls. However, these decreases in enzyme activity were accompanied by a decrease in template activity only for acetyl-CoA carboxylase, further suggesting differences in the role that protein synthesis plays in the regulation of these two enzymes. The injection of
Table 2. *Induction of acetyl-CoA carboxylase and HMG-CoA reductase in streptozotocin-diabetic rats*

Rats were made diabetic by a single intraperitoneal injection of streptozotocin (80 mg/kg body wt.) and the onset of diabetes mellitus was monitored by measurement of serum glucose. Insulin was administered as a single intraperitoneal injection (10 units/kg body wt.). Rats given fructose were fed with a high-fructose diet for 7 days before use in this experiment. Data are given as means ± S.D. of duplicate determinations on four different animals in each experimental group: significant differences from the control are indicated by *P < 0.01 and **P < 0.005.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetyl-CoA carboxylase (units/mg of protein)</th>
<th>10^4 × Template activity</th>
<th>HMG-CoA reductase (units/mg of protein)</th>
<th>10^4 × Template activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.28 ± 0.05</td>
<td>0.08 ± 0.02</td>
<td>0.12 ± 0.03</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.68 ± 0.10*</td>
<td>0.05 ± 0.01*</td>
<td>0.07 ± 0.02*</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Diabetic + insulin</td>
<td>3.48 ± 0.43**</td>
<td>0.29 ± 0.05**</td>
<td>0.55 ± 0.17**</td>
<td>0.19 ± 0.03*</td>
</tr>
<tr>
<td>Diabetic + fructose</td>
<td>3.49 ± 0.34**</td>
<td>0.17 ± 0.02*</td>
<td>0.49 ± 0.02**</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
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References


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