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Synthesis and Degradation of Liver Acetyl Coenzyme A Carboxylase in Genetically Obese Mice

(increased hepatic lipogenesis/immunochemical analysis/Michaelis constants/ heat stability/[^{3}H]leucine incorporation)

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ABSTRACT **The total cytosol activity of acetyl-CoA carboxylase (acetyl-CoA:CO_2 ligase (ADP), EC 6.4.1.2) in the liver is known to be 6- to 10-fold higher in genetically obese hyperglycemic mice (C57BL/6J-*ob*) than in nonobese mice. The results of immunochemical titrations, Ouchterlony double-diffusion analysis, and kinetic and heat inactivation studies indicated that this rise in the level of carboxylase activity in liver extracts from obese mice was ascribed to an increase in the quantity of the enzyme protein, which was indistinguishable from that derived from nonobese mice. Combined immunochemical and isotopic techniques showed that the rate of synthesis of the carboxylase per liver was 7.7-fold higher in obese than in nonobese mice. The rate of degradation of the carboxylase was found to be 1.7-fold lower in obese than in nonobese mice, the half-life being 115 and 67 hr, respectively. These results indicate that the increase in the acetyl-CoA carboxylase content of the liver in obese mice is due mainly to a rise in the rate of enzyme synthesis, and in a minor degree, to a decrease in the rate of enzyme degradation.**

Genetically obese hyperglycemic mice (C57BL/6J-*ob*) carry a single recessive mutant gene for obesity (1). Although the basic cause of this obesity remains unexplained, much is known about the metabolic derangements that occur in these mice. One of the most significant metabolic abnormalities is a remarkable increase in hepatic lipogenesis (2) that results from higher levels of several enzymes involved in long-chain fatty acid synthesis, including acetyl-CoA carboxylase (3), fatty acid synthetase (3), and citrate cleavage enzyme (4). These studies on the lipogenic enzymes, however, were based on measurements of catalytic activities that do not answer the question whether the mutation affects the structure of the enzyme(s) or alters the regulation of synthesis and degradation of the enzyme(s).

Acetyl-CoA carboxylase plays a critical role in the regulation of long-chain fatty acid synthesis (5). Immunochemical studies performed recently by our group (6, 7) and by Majerus and Kilburn (8) have disclosed that the changes in the level of acetyl-CoA carboxylase in liver extracts derived from rats under different dietary and hormonal conditions are determined by changes in the quantity of the carboxylase protein. It has been further shown that the increase or decrease in the enzyme quantity in refed or alloxan-diabetic rats is due to changes in the rate of enzyme synthesis, while the decrease in the enzyme quantity in fasted rats is due to both diminished enzyme synthesis and accelerated enzyme degradation.

In the present work, an attempt has been made to define the mechanisms underlying the increase in the level of liver acetyl-CoA carboxylase in obese mice by means of immunochemical and kinetic methods. The antibody prepared against homogeneous acetyl-CoA carboxylase from rat liver (6, 7) was found to crossreact with the enzyme from mouse liver.

MATERIALS AND METHODS

Animals, chemicals, and determinations

Obese hyperglycemic mice (C57BL/6J-*ob*) and their nonobese littermates were purchased from the Jackson Memorial Laboratory, Bar Harbor, Me. Mice of the C57BL/6J strain used as nonobese controls were a gift of Takeda Chemical Industries, Osaka, Japan. Male obese and nonobese mice, 10–14 weeks of age, were used in all experiments, except that both male and female nonobese mice were used to prepare the carrier enzyme for studies on the incorporation of [^{3}H]leucine. Mice were fed *ad libitum* a balanced diet obtained from Clea, Tokyo, Japan. All chemicals and determinations used were as described in a previous report (7).

Enzyme assay

Activity of acetyl-CoA carboxylase was determined at 37°C by the $H^{14}CO_3^-$-fixation assay as described previously (7). One unit of the enzyme is defined as that amount which catalyzes the carboxylation of 1 μmol of acetyl-CoA per minute at 37°C under the conditions used.

Immunochemical procedures

Antibody against acetyl-CoA carboxylase from homogeneous rat liver was prepared as reported earlier (7). The procedures for immunochemical titrations, Ouchterlony double-diffusion analysis, quantitative precipitin reactions, and [^{3}H]-leucine incorporation studies were described (7).

Enzyme preparations

All manipulations were performed at 0–4°C. The method of preparing the enzyme that was used for kinetic studies was similar to that described previously to obtain enzyme preparations used for quantitative precipitin reactions (7), except that the final DEAE-cellulose eluate containing the carboxylase was concentrated by precipitation with ammonium sulfate (33% saturation). This procedure yielded 20- to 30-fold purified enzyme, as compared to the liver extract obtained by centrifugation at 105,000 $\times$ *g*.

For heat inactivation studies, the enzyme was purified further by fractionation on calcium phosphate gel as follows. The DEAE-cellulose eluate was adsorbed on calcium phosphate gel (protein:gel = 1:2). The gel was washed three times with 33 mM potassium phosphate buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and 1 mM

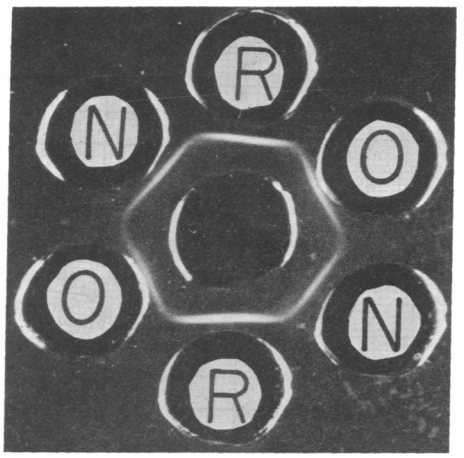

FIG. 1. Ouchterlony double-diffusion analysis of acetyl-CoA carboxylase from livers of rats, obese mice, and nonobese mice. Agar gel (1.0%) contained 20 mM Tris·HCl buffer (pH 7.5), 0.15 M NaCl and 0.1% sodium azide. The plate was developed at 4°C for 2 days before the photograph was taken. The center well contained antiacetyl-CoA carboxylase γ-globulin; wells *R*, *O*, and *N* contained partially purified enzyme preparations from rats, obese and nonobese mice, respectively. Other details were as described (7).

EDTA, and then eluted twice with 0.2 M potassium phosphate buffer (pH 7.5) containing the same components. The combined eluates that contain the carboxylase were concentrated as described above. The enzyme preparation thus obtained was purified about 100-fold compared to the liver extract obtained by centrifugation at 105,000 × *g*.

RESULTS

Characterization of antibody

Since it was difficult to obtain sufficient quantities of homogeneous acetyl-CoA carboxylase from mouse liver to be used as antigen, the crossreactivity of antibody prepared against homogeneous acetyl-CoA carboxylase from rat liver with the mouse liver enzyme was examined. Fig. 1. shows an Ouchterlony double-diffusion pattern of this antibody in the center well and carboxylase preparations obtained from livers of rats, obese mice, and nonobese mice. The completeness of connections of the precipitin bands revealed that carboxylase molecules derived from the three sources were immunologically similar to each other.

The results of quantitative precipitin reactions with enzyme preparations obtained from livers of rats and nonobese mice are represented in Fig. 2. When the enzyme activity remaining in the supernatant solution after removal of the enzyme–antibody complex was measured, identical equivalence points for the enzymes from the two sources were found. Furthermore, the protein content of the antigen–antibody precipitates was similar for both enzymes, when equivalent amounts of enzyme activities were added. These findings indicate a high degree of crossreactivity of the two enzymes with the antiacetyl-CoA carboxylase used and justify the use of this antibody for the present studies.

Immunochemical titration

The level of acetyl-CoA carboxylase in liver extracts from obese mice, which were 10–14 weeks of age and weighed 39–44 g, was compared with that in liver extracts from nonobese mice, which

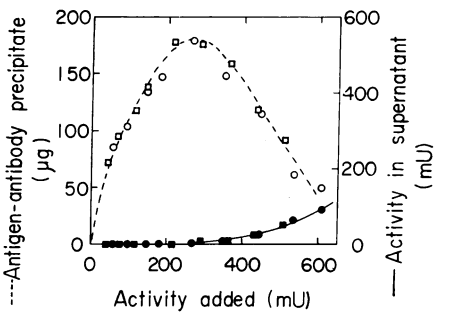

FIG. 2. Quantitative precipitin reactions of acetyl-CoA carboxylase from livers of rats and nonobese mice. Increasing amounts of partially purified enzyme preparations containing the carboxylase activities indicated were added to 1.1 mg of anti-acetyl-CoA carboxylase γ-globulin. Protein precipitate, rats (□), nonobese mice (○); enzymeactivity remaining in supernatant solution, rats (■), nonobese mice (●).

were of the same age and weighed 22–26 g. The specific activity was 3- to 4-fold higher in obese than in nonobese mice, while the total liver cytosol activity was increased 6- to 10-fold in obese mice. These results were in general agreement with those reported by Chang *et al.* (3). In our experiments, mice of the C57BL/6J strain were used as nonobese controls, while these investigators used nonobese littermates that were genotypically either homozygous (+/+) or heterozygous (*ob*/+). However, we found no significant difference in either the specific activity or the total activity of the carboxylase between the C57BL/6J strain of mice and the nonobese littermates.

In order to decide whether the difference in the level of liver acetyl-CoA carboxylase between obese and nonobese mice is due to a change in catalytic efficiency per enzyme molecule or to a change in the number of enzyme molecules, i.e. enzyme quantity, immunochemical titrations of liver extracts from both mice were performed. The results represented in Fig. 3 showed that the equivalence point was the same for both liver extracts when based on the amount of enzyme activity added, indicating that the change in the level of activity was accompanied by a proportionate change in the quantity of immunochemically reactive protein.

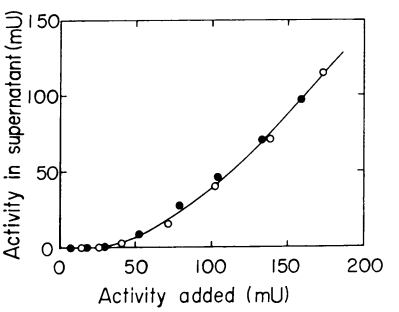

FIG. 3. Immunochemical titration of acetyl-CoA carboxylase from livers of obese and nonobese mice. Increasing amounts of gel-filtered liver extracts containing the carboxylase activities indicated were added to 90 μg of antiacetyl-CoA carboxylase γ-globulin (specific activity of the enzyme preparation from obese mice, 21.0 munits/mg of protein; from nonobese mice, 7.0 munits/-mg of protein). Obese mice (○), nonobese mice (●).

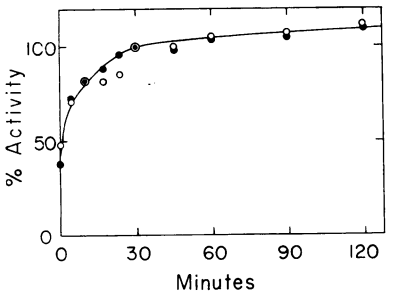

FIG. 4. Preincubation with citrate of acetyl-CoA carboxylase from livers of obese and nonobese mice. The preincubation mixtures contained in a total volume of 1.0 ml: 50 μmol of Tris·HCl buffer (pH 7.5), 10 μmol of $MgCl_2$, 3.75 μmol of GSH, 750 μg of bovine serum albumin, 10 μmol of potassium citrate, and 180 μg of partially purified enzyme (see *Materials and Methods*) either from obese mice (○) or from nonobese mice (●). Assay of carboxylase activity was performed as described in *Materials and Methods*, except that the time of preincubation at 37°C was varied as indicated. The values obtained after preincubation for 30 min were taken as 100% and used as reference for the calculation of all other points.

Kinetic properties and heat stability

Some properties of partially purified acetyl-CoA carboxylase preparations obtained from livers of obese and nonobese mice were compared to examine further whether or not the enzymes from both sources exhibit qualitative differences. For full activation of the mouse liver carboxylase, citrate was found to be required both during preincubation and during the carboxylation reaction, as reported previously with the rat liver enzyme (9). Fig. 4 illustrates the effect of preincubation with citrate on enzyme activity. The time course of the activation was similar with the enzymes from both sources. The effects on enzyme activity of various concentrations of ATP, acetyl-CoA, and citrate during the reaction were also studied, and the Michaelis constants (K_m) found are summarized in Table 1. There were no significant differences in the K_m values of the enzymes from the two sources. Table 2 shows the effect on enzyme activity of various concentrations of palmityl-CoA during the reaction. Both enzymes were

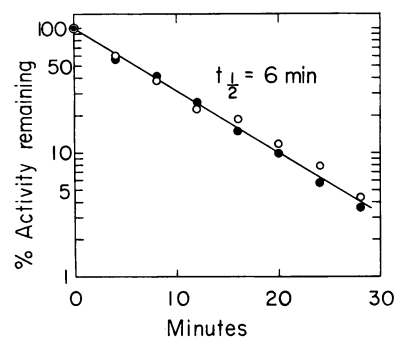

FIG. 5. Heat inactivation of acetyl-CoA carboxylase from livers of obese and nonobese mice. The preincubation mixtures contained in a total volume of 0.3 ml: 15 μmol of Tris·HCl buffer (pH 7.5), 3 μmol of $MgCl_2$, 1.12 μmol of GSH, 225 μg of bovine serum albumin, and 45 munits of partially purified enzyme (see *Materials and Methods*) either from obese (○) or from nonobese mice (●). The mixtures were heated at 45°C, and aliquots were removed for assay of carboxylase activity at the indicated times.

TABLE 1. *Michaelis constants of acetyl-CoA carboxylase from livers of obese and nonobese mice*

	K_m (M)	
	Nonobese	Obese
ATP	1.2×10^{-5}	1.2×10^{-5}
Acetyl-CoA	1.9×10^{-5}	3.2×10^{-5}
Citrate	5.0×10^{-3}	4.3×10^{-3}

The values were obtained according to Lineweaver-Burk plots. For the enzyme preparation used, see *Materials and Methods*.

almost equally inhibited by this long-chain acyl-CoA derivative that can be regarded as an endproduct inhibitor (10).

When the enzymes from obese and nonobese mice were heated at 45°C, essentially identical rates of inactivation were observed, with a $t_{1/2}$ of 6 min, as illustrated in Fig. 5. The results of the kinetic and heat inactivation studies, together with those of the Ouchterlony double-diffusion analysis and the immunochemical titrations, lead to the conclusion that the increased level of carboxylase activity in liver extracts from obese mice is actually determined by an increased quantity of the enzyme protein indistinguishable from that derived from nonobese mice.

Rates of synthesis and degradation

Since the amount of an enzyme represents a steady state achieved by a balance between the rate of synthesis and the rate of degradation, a mutation affecting either or both rates could be responsible for the increased content of acetyl-CoA carboxylase in livers of genetically obese mice. In order to distinguish among these possibilities, experiments were performed to estimate the rates of synthesis and degradation of acetyl-CoA carboxylase in livers of obese and nonobese mice.

As a measure of the rate of enzyme synthesis, the extent of [^{3}H]leucine incorporation into the protein precipitated by antibody against acetyl-CoA carboxylase after pulse-labeling was determined. The results of such experiments with obese and nonobese mice are shown in Table 3. Since the extent of labeling of total soluble liver protein is reflected in that of the carboxylase, the ratio of the radioactivity incorporated into the enzyme to that incorporated into total soluble protein (a/b) was calculated. This relative rate of enzyme synthesis

TABLE 2. *Inhibition by palmityl-CoA of acetyl-CoA carboxylase from livers of obese and nonobese mice*

Palmityl-CoA (M)	Activity (%)	
	Nonobese	Obese
0	100	100
1×10^{-6}	86.7	88.0
5×10^{-6}	56.0	47.9
1×10^{-5}	35.1	32.4
4×10^{-5}	14.2	7.6
8×10^{-5}	1.8	1.3

Acetyl-CoA carboxylase was assayed as described in *Materials and Methods*, except that the assay mixture contained palmityl-CoA as indicated.

TABLE 3. *Relative rates of acetyl-CoA carboxylase synthesis in livers of obese and nonobese mice*

Mice	Mean weight of mouse (g)	Mean weight of liver (g)	Acetyl-CoA carboxylase in liver extract: Specific activity (munits/mg)	Acetyl-CoA carboxylase in liver extract: Total activity per liver (units)	[^{3}H]Leucine incorporation: (*a*) acetyl-CoA carboxylase per liver (cpm)	[^{3}H]Leucine incorporation: (*b*) Total soluble protein (cpm/mg)	*a/b*
Nonobese	24	1.18	4.7	0.36	1,830	15,100	0.12
Obese	40	3.40	16.7	3.66	15,800	16,900	0.93

Obese mice weighing 39–41 g were each injected once, intraperitoneally, with 720 μCi of [4,5-^{3}H]L-leucine (17 Ci/mmol) dissolved in 0.8 ml of 0.15 M NaCl. Nonobese mice weighing 23–25 g were each injected once, intraperitioneally, with 360 μCi of the same labeled amino acid dissolved in 0.4 ml of 0.15 M NaCl. 1.5 hr later, three obese mice or 10 nonobese mice were killed, and the radioactivity in both acetyl-CoA carboxylase in the liver, precipitated by antiacetyl-CoA carboxylase, γ-globulin and total soluble liver protein, precipitated by trichloroacetic acid, were determined (7). The extent of the nonspecific precipitation of radioactivity in the enzyme–antibody precipitates was less than 20% of the total radioactivity in the precipitates.

per liver was 7.7-fold higher in obese mice than in nonobese mice. In terms of the rate of enzyme synthesis per gram of liver, this increase was 2.7-fold.

As a measure of the rate of enzyme degradation, the rate of loss of radioactivity from the labeled carboxylase was determined after injection of mice with [^{3}H]leucine. The data are represented in Fig. 6. The decay of specific radioactivity of the carboxylase in both mice appeared to follow a first-order reaction. The rate of loss of isotope, expressed as half-life ($t_{1/2}$), was 115 hr in obese mice and 67 hr in nonobese mice, so that the rate of enzyme degradation was decreased 1.7-fold in obese animals.

The results of the studies on labeled leucine incorporation indicate that the increase in the amount of the carboxylase in livers of obese mice is due mainly to a rise in the rate of enzyme synthesis, and in a minor degree, to a fall in the rate of enzyme degradation. The possibility remains, however, that the prolonged half-life of liver acetyl-CoA carboxylase in obese mice may result from a higher degree of reutilization of [^{3}H]leucine in these animals. Although the use of guanido-labeled arginine, which is not reutilized (11), was desirable, it was impractical to use this labeled amino acid because large doses were required for our experiments. As shown in Fig. 6, there was no essential difference in the half-life of total soluble liver protein between obese mice ($t_{1/2}$ = 124 hr) and nonobese mice ($t_{1/2}$ = 109 hr). Thus, it appears reasonable to conclude that the half-life of liver acetyl-CoA carboxylase is actually prolonged in obese mice.

DISCUSSION

Under steady-state conditions, the content of an enzyme is related to the rates of its synthesis and degradation as follows. $E = k_s/k_d$, where E is the enzyme content per mass, k_s is a zero-order rate constant of synthesis per mass, and k_d is a first-order rate constant of degradation, expressed as the reciprocal of time (12). Since, in obese mice, the relative rate of enzyme synthesis per liver (or per gram of liver) was increased 7.7-fold (or 2.7-fold) and the rate constant of enzyme degradation was decreased 1.7-fold, it is predicted from the above equation that there would be a 13.1-fold (or 4.6-fold) increase in the enzyme content per liver (or per gram of liver) in obese mice. These valu esagree fairly well with the 10.1-fold (or 3.5-fold) difference in the enzyme content per liver (or per gram of liver) actually found between obese and nonobese mice (see Table 3).

Of the biochemical mutations encountered in inbred mouse strains, some affect the structure of an enzyme, while others alter the tissue concentration of an enzyme (12). The immunochemical, kinetic, and heat inactivation studies presented suggest that the liver acetyl-CoA carboxylase derived from genetically obese mice has the same structure as that derived from nonobese mice. Rechcigl and Heston (13) and Ganschow and Schimke (14) reported that among certain sublines of the C57BL strain, a mutation occurs that affects the content of liver catalase by changing the rate of enzyme degradation. On the other hand, Doyle and Schimke (15) described another mutation that affected the concentration of liver δ-aminolevulinate dehydratase by altering the rate of enzyme synthesis. The present results indicate that in genetically obese mice, the content of liver acetyl-CoA carboxylase is affected by changes in both the rate of enzyme synthesis and the rate of enzyme degradation. It remains

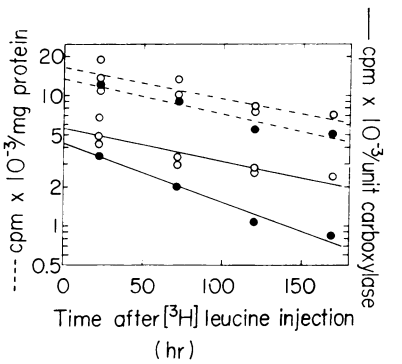

FIG. 6. Kinetics of degradation of acetyl-CoA carboxylase and total soluble protein in livers of obese and nonobese mice. At zero time, obese mice weighing 39–42 g and nonobese mice weighing 22–26 g were each given a single intraperitoneal injection of [^{3}H]leucine as described for Table 3. Two obese mice or 10 nonobese mice were killed at the indicated times. Further details were the same as given for Table 3. Radioactivity per unit of acetyl-CoA carboxylase, obese mice (O——O), nonobese mice (●——●); radioactivity per mg of total liver soluble protein, obese mice (O- - -O), nonobese mice (●- - -●).

unknown, however, whether these changes in the rates of synthesis and degradation of the carboxylase are the primary error resulting from the mutation or secondary to other metabolic or hormonal derangements caused by the mutation.

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