Age-Related Changes in Rat Hepatic Acetyl-Coenzyme A Carboxylase (44560)

K. GEORGE THAMPY, MICHAEL J. HAAS, AND ARSHAG D. MOORADIAN[1]

Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Saint Louis University School of Medicine, St. Louis, Missouri

Abstract. **Acetyl-CoA carboxylase (ACC) catalyzes the rate-limiting step in the synthesis of long-chain fatty acids. Since aging influences adiposity, we studied the activity of ACC and its mRNA content in livers of 4-, 12-, and 24-month-old male Fischer 344 rats. The mean (± SEM) activity of ACC (mU/mg protein) in liver homogenates from 4-month-old rats was 1.01 ± 0.14. There was an 80% increase in activity (1.83 ± 0.27) in 12-month-old rats ($P < 0.01$). However, there was significantly less activity (0.46 ± 0.06) in livers of 24-month-old rats ($P < 0.001$). The total activity of ACC (per g liver) followed the same trend. The enzyme from all age groups was purified by avidin-affinity chromatography. The purified preparation migrated as a major protein band (M_r 262,000) on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The specific activity of the purified preparation was 1.5, 1.8, and 1.8 U/mg for 4-, 12-, and 24-month-old rats, respectively. The alkali-labile phosphate content was 5.66 ± 0.17, 5.64 ± 0.21, and 6.21 ± 0.35 mols P_i/mole subunit for 4-, 12-, and 24-month-old rats, respectively. These age-related differences were not significant. The hepatic ACC mRNA measured by ribonuclease protection assay when corrected for G3PDH mRNA was significantly reduced in 24-month-old rats (0.24 ± 0.03) compared with 12-month-old (0.58 ± 0.04) or 4-month-old rats (0.43 ± 0.007) $P < 0.01$. In summary: (i) Aging in rats is associated with significant changes in ACC activity; (ii) the purified ACC preparations from the three age groups had similar specific activity and similar phosphate content; and (iii) the changes in ACC mRNA content of the liver paralleled the changes in total enzyme activity when 12-month-old rats were compared with 24-month-old rats whereas the increase in ACC activity in 12-month-old rats compared with 4-month-old rats could not be ascribed to changes in hepatic mRNA levels. These results indicate that the age-related changes in hepatic ACC occur at a post-translational level during early years of aging and at a pretranslational level at late states of senescence. These changes may contribute to the age-related alterations in body adiposity.**

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Acetyl-CoA carboxylase (ACC) catalyzes the formation of malonyl-CoA from acetyl-CoA, the rate-limiting and first committed step in the formation of long-chain fatty acids (1). The mammalian liver enzyme is composed of multiple (>40) identical subunits with molecular weight (1) of 265 kDa. Each subunit has a biotin prosthetic group, two catalytic activities, namely biotin carboxylase and transcarboxylase, and the allosteric site for citrate.

ACC is a highly regulated enzyme. The acute control of ACC involves covalent modification and allosteric mechanisms. The enzyme is known to be interconverted by phosphorylation-dephosphorylation mechanisms. The phosphorylated form is less active whereas the dephosphorylated form is the more active form (2). The enzyme is subject to allosteric control by citrate that at physiological concentrations stimulates the activity of ACC (3). Whereas allosteric control occurs within short time periods, the long-term control of ACC involves changes in the specific mRNA levels and the rate of protein synthesis and degradation. The mechanisms involved are complex and not clearly known.

Aging is known to alter adiposity and adipogenic po-

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[1] To whom requests for reprints should be addressed at the Division of Endocrinology, Saint Louis University, 3691 Rutger, Suite 101, St. Louis, MO 63110. E-mail: mooradad@SLU.EDU

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tential in mammals (4). In humans, there is a gradual increase in body adiposity that peaks in late adulthood; thereafter, loss of body mass may occur at later stages of life (5). The changes in body adiposity of Fischer 344 rats are similar to the changes in humans (6). The underlying mechanisms of these age-related changes are multiple. One potential mechanism is altered expression of lipogenic enzymes (7–9). In this communication, we report on the age-related changes in the expression and activity of ACC, the rate-limiting enzyme of long-chain fatty acid synthesis.

Materials and Methods

Experimental Animals. Fischer 344 (F344) male rats of various age groups were obtained from Harlan Industries, (Indianapolis, IN). The rats were fed regular laboratory rat chow until they were sacrificed by exsanguination through the abdominal aorta under brief sodium pentobarbital anesthesia (45 mg/kg ip). The body weight and food intake of the rats were monitored every other day for 10 days before the experiments. The livers were obtained and frozen immediately in liquid nitrogen and stored at −80°C until used. Rats were inspected for gross pathology of internal organs. Those having tumors or poor food intake were excluded from the study. The protocol for handling and sacrificing the rats was approved by the Saint Louis University Animal Care Committee.

Chemicals and Reagents. [^{14}C]bicarbonate was obtained from ICN Radiochemicals (Costa Mesa, CA). Acetyl-CoA, avidin, cyanogen bromide, polyethylene glycol 8000, biotin, benzamidine, leupeptin, and aprotinin were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Liver Fractions. Frozen livers were homogenized in 10 volumes of homogenization buffer (50 m*M* potassium phosphate, pH 7.4, 1.0 m*M* ethylenediaminetetraacetic acid (EDTA), 2 m*M* dithiothreitol (DTT), 5 mg/l aprotinin, 5 mg/l leupeptin and 2 m*M* benzamidine using a Polytron homogenizer (Westbury, NY) for 20 sec. The temperature throughout the procedure was kept between 0°C and 4°C. Subsequently this homogenate was centrifuged at 15,000*g* for 20 min, and the supernatant was filtrated through glass-wool and collected.

Purification of Acetyl-CoA Carboxylase. Livers from four to five animals from each group were used for purification. The enzyme was purified from frozen samples by polyethylene glycol fractionation and avidin-Sepharose chromatography as described previously (3, 10). The enzyme was eluted using 0.1 m*M* biotin solution. Ammonium sulfate was added to the pooled fractions to a final concentration of 25% saturation. The precipitated protein was collected by centrifugation at 15,000*g* for 20 min and resuspended in column buffer (0.5 *M* NaCl, 0.1 *M* Tris. HCl, pH 7.5, 0.1 m*M* EDTA, 0.1 m*M* DTT, and 5% glycerol). Polyacrylamide gel (5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) showed one major band at MW 262 kDa (Fig. 1). The purity of various preparations was similar based on their electrophoretic mobility in such gels.

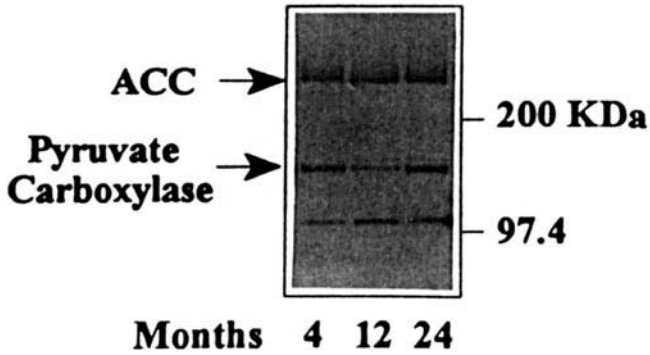

Figure 1. A representative 5% sodium dodecyl sulfate polyacrylamide gel showing the electrophoretic mobility of purified acetyl CoA carboxylase enzyme from the liver of 4-, 12-, and 24-month-old rats. The expected molecular weight of the purified enzyme subunit is shown at 262 kDa. The other bands represent other biotenylated enzymes such as pyruvate carboxylase (≈ 130 kDa).

These experiments were done twice with different sets of animals.

Protein Determination. The protein was determined by the bicinchoninic acid method according to the manufacturer's recommendations (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the standard.

Assay of Acetyl-CoA Carboxylase. The assay measured incorporation of [^{14}C]bicarbonate into malonyl-CoA (3, 10). Each assay tube contained crude protein preparation (0.1–0.2 mg) from liver or affinity purified enzyme (0.1–0.2 μg). The assay mixture contained 50 m*M* HEPES, pH 7.5, 2.5 m*M* $MnCl_2$, 2.0 m*M* dithiothreitol, 0.125 m*M* acetyl-CoA, 4.0 m*M* ATP, 12.5 m*M* [^{14}C]potassium bicarbonate (2×10^6 cpm), 0.75 mg/ml bovine serum albumin and 10 m*M* citrate in a final volume of 0.15 ml. One unit of activity is defined as 1 μmol of malonyl-CoA formed per min at 37°C. For this assay, 10 4-month-old rats, 5 12-month-old rats, and 5 24-month-old rats were used.

Phosphate Determination. Alkali-labile phosphate was determined after a 3-hr hydrolysis of the affinity-purified protein fraction (20–40 μg) in the presence of 1.0 *N* sodium hydroxide (NaOH) at 100°C for 4 hr. At the conclusion of hydrolysis, the NaOH was neutralized with 10 *N* sulfuric acid, and the phosphate was estimated colorimetrically as previously described (11).

Ribonuclease Protection Assay. Total RNA was isolated from 100 mg of frozen liver tissue using the one-step acid guanidinium thiocyanate-phenol-chloroform extraction as described previously (12) and resuspended in diethylpyrocarbonate-treated water. The ribonuclease protection assay was performed with a Kit (Multi-NPA, Ambion, Austin, TX). An antisense oligonucleotide probe complementary to nucleotides 2781–2820 of ACC cDNA (5′-AATCTGCTGGCTGGGAAACTGACACAG-GACCGATGTGAT (Genbank accession number J03808) and containing a five-nucleotide noncomplementary extension GAACGA-3′ was synthesized and labeled to high specific activity with [γ^{32}P]-ATP and T4-polynucleotide kinase (13). Yeast RNA was included as a control for hybrid-

ization specificity and to verify complete digestion of the nonhybridized probe (14). Five μg of RNA were mixed with 25,000 cpm of DNA probe, coprecipitated with 2.5 volumes of absolute ethanol after adjusting the mixture to contain 0.5 *M* ammonium acetate, and placed at −20°C for 15 min. The precipitated material was collected by centrifugation at 10,000*g* for 15 min, washed once with 70% ethanol, dried in a vacuum, and resuspended in 10 μl of hybridization buffer. The samples were incubated overnight at 31°C, then incubated in the proprietary nuclease mixture (Ambion) (diluted 1:200, v/v) at 31°C for 30 min. After addition of the stop mix (Ambion), the DNA protected from digestion was recovered by precipitation with ethanol, dried in a vacuum, and resuspended in the gel-loading mix (Ambion). Samples were fractionated on an 8% polyacrylamide sequencing gel. The gel was fixed in 5% acetic acid, 5% methanol, dried, and exposed to film for 6–16 hr. The amount of signal was quantitated with a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA). All the signals acquired were within the linear range of the ribonuclease protection assay calibration curves. A similar procedure was used to examine glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) expression as a control. The rat G3PDH oligonucleotide (Ambion) was labeled with ^{32}P and T4-polynucleotide kinase as described above. The probe (25,000 cpm) was mixed with the liver RNA, precipitated with ethanol, resuspended in hybridization buffer (Ambion), and incubated at 37°C overnight. The samples were incubated in the nuclease mixture (1:200, v/v) for 30 min at 37°C. The remaining steps in the analysis were identical to those described above.

Statistical Analysis. The results are reported as mean ± SEM. Two-tailed Student's *t* test with Bonferroni's correction was used to determine the statistical significance of the difference. A $P < 0.05$ was considered the limit of statistical significance.

Results

The body weight and daily food intake of the rats were monitored for 10 days. The results were published previously (9). Briefly, the daily food intake (g/day/rat) of 4-, 12-, and 24-month-old rats was 18.9 ± 2.3, 16.8 ± 1.9 and 14.1 ± 2.5, respectively. Overall, 24-month-old rats consumed fewer calories than younger rats ($P < 0.01$).

The hepatic ACC activity in mU/mg protein was 1.01 ± 0.14, 1.83 ± 0.27, and 0.46 ± 0.06 in 4-, 12-, and 24-month-old rats, respectively. The activity expressed as mU/g liver was 130 ± 18, 234 ± 34, and 57 ± 10, respectively. The increase in ACC activity observed in 12-month-old rats and subsequent suppression in 24-month-old rats compared with 4-month-old rats were statistically significant, $P < 0.001$ (Fig. 2). The total liver weight of 24-month-old rats is modestly larger than that of 12-month-old rats (15). This difference does not abrogate the significant decrease in ACC activity in 24-month-old rats.

Purified ACC from different age groups was compared.

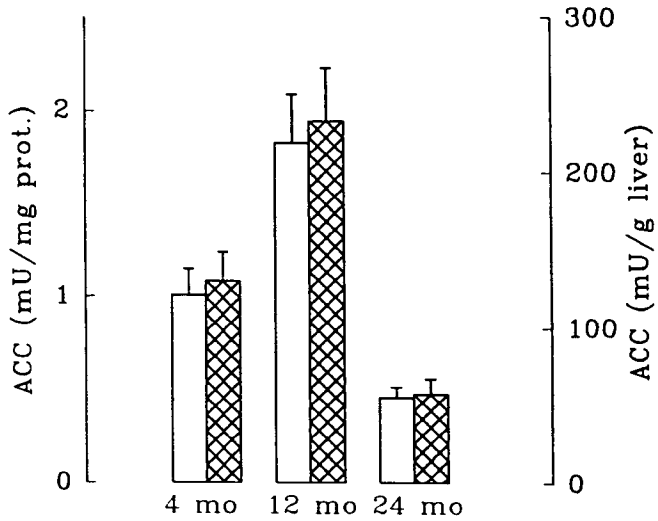

Figure 2. The mean (±SEM) of acetyl-CoA carboxylase (ACC) activity in rat liver homogenates from different age groups. ACC activity was assayed in rat liver homogenates (15,000*g* supernatant) by the carbon dioxide fixation method (3, 10). One unit of activity is defined as 1 μmol of malonyl-CoA formed per min at 37°C. Clear boxes represent activity per mg protein, and hatched boxes represent activity per g liver. $n = 10$ for 4-month-old, $n = 5$ for 12- and 24-month-old. $P < 0.01$.

As expected, the purified preparations migrated as a major protein band of MW 262,000 daltons on polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1).

The specific activity of the purified preparations as a function of citrate concentration is shown in Figure 3. It is evident that all preparations had negligible activity when assayed in the absence of citrate, but they were activated by the presence of citrate in a concentration-dependent manner. The purified ACC from rats of various age groups had comparable activity in the presence of 10 m*M* citrate. However, when the enzyme was assayed at 0.1–0.5 m*M* citrate (physiological citrate level) (16), the ACC preparation from 12-month-old rats (0.557 ± 0.112 U/mg) had ≈ 5-fold higher activity than 4 (0.106 ± 0.033U/mg) or 24 (0.106 ± 0.029U/mg)-month-old rats.

To characterize the purified preparations further, alkali-labile phosphate content of ACC was measured. The phosphate content of purified enzyme (mol P_i/mol subunit) was 5.66 ± 0.17, 5.64 ± 0.21, and 6.21 ± 0.35 in 4-, 12-, and 24-month-old rats, respectively. There were no significant age-related differences in phosphate content.

To determine if changes in enzyme activity occur at a pretranslational level, acetyl-CoA carboxylase mRNA content was measured using a quantitative ribonuclease protection assay (RNP) (14). A representative gel of RNP is shown in Figure 4. Hepatic acetyl CoA carboxylase mRNA content when normalized to G3PDH mRNA was 0.43 ± 0.07, 0.58 ± 0.04, and 0.24 ± 0.03 in 4-, 12-, and 24-month-old rats, respectively. The decrease in hepatic ACC mRNA content of the 24-month-old group compared with the 12-month-old or 4-month-old group was significant ($P < 0.001$). These results suggest that the decrease in enzyme

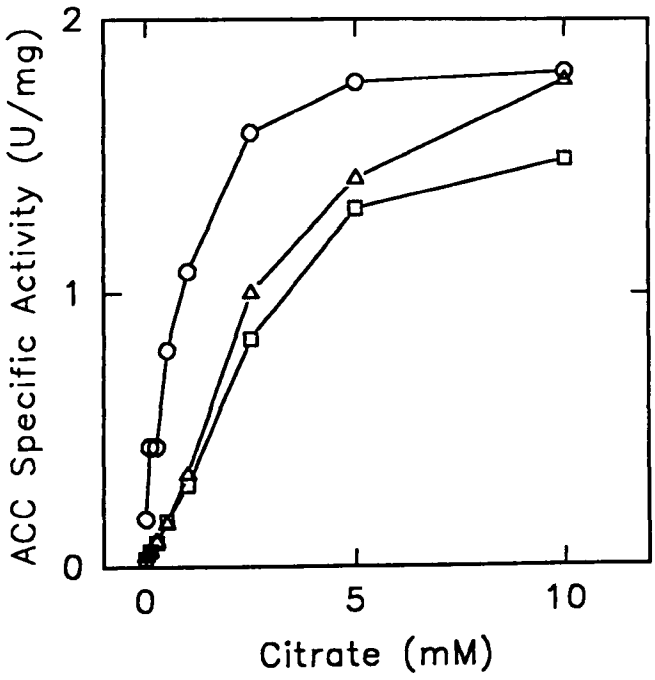

Figure 3. Citrate dependence of acetyl-CoA carboxylase (ACC) activity in various preparations purified from the liver of rats at 4 (□—□), 12 (○—○), and 24 months of age (△—△). ACC from rat liver was purified by avidin-affinity chromatography. The purified preparation was assayed for ACC in the presence of various concentrations of citrate. The increased citrate sensitivity of ACC from 12-month-old rats is evident.

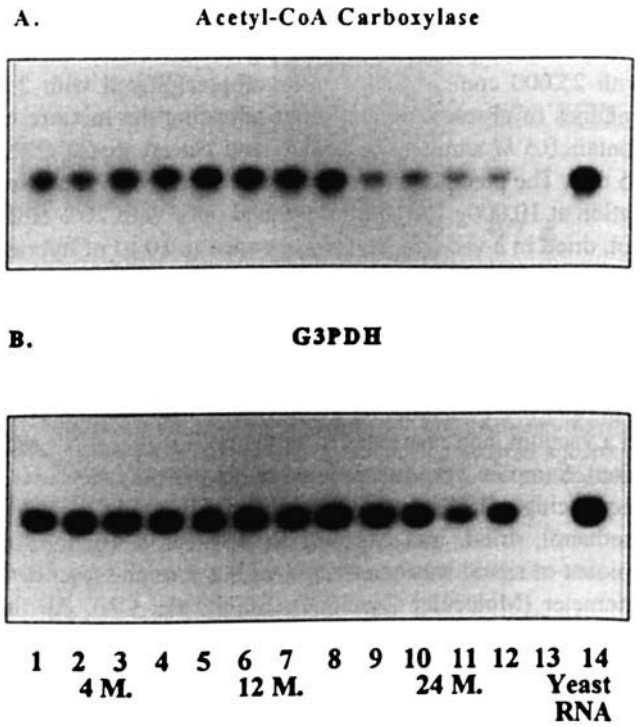

Figure 4. Quantitative ribonuclease protection assay to measure acetyl-CoA carboxylase mRNA content. ACC mRNA in the liver (A) of male rats of 4 (Lanes 1–4), 12 (Lanes 5–8), and 24 months of age (Lanes 9–12). Five μg of total liver RNA was mixed with either an antisense probe specific for (A) the acetyl-CoA carboxylase message or (B) the G3PDH message. The slight increase observed in 12-month-old animals is not statistically significant when normalized to the G3PDH control. The repression observed in the 24-month-old animals is statistically significant ($P < 0.001$). Lanes 13 and 14 contain yeast RNA with and without nuclease, respectively, as internal negative control.

activity observed in 24-month-old rats is due primarily to a decrease in mRNA concentrations. However, the rise in enzyme activity at 12 months of age compared with 4-month-old rats is probably independent of the changes in mRNA content.

Discussion

Acetyl-CoA carboxylase (ACC) catalyzes the first committed step in the synthesis of fatty acids (1). This reaction is also the rate-limiting step in fatty acid synthesis and probably has a pivotal role in fat metabolism (17–20). The results of this study indicate that the activity of liver ACC varies with age. The enzyme activity peaks in 12-month-old rats but is significantly reduced in 24-month-old rats. This suggests that fatty acid synthesis may be increased in middle-aged rats but may be reduced with further senescence.

To understand the molecular mechanisms underlying these changes, we studied the enzyme in crude as well as affinity-purified preparations and quantitated the amount of specific mRNA in samples of liver obtained from different age groups. The specific activity of the purified preparation, assayed in the presence of 10 m*M* citrate, did not show any changes as a function of aging. However, the preparation from 12-month-old rats showed 4–5-fold higher specific activity when assayed in the presence of physiological concentrations of citrate (0.1–0.5 m*M*). This implies that the enzyme from 12-month-old rats is more sensitive to citrate at physiological concentrations.

ACC is known to be regulated by phosphorylation/dephosphorylation (18–20). The phosphorylated form is the inactive form whereas the dephosphorylated form is the active one. In addition, the enzyme has a number of phosphorylation sites with varying selectivity for modulation of enzyme activity. For example, dephosphorylation of certain sites is known to increase selectively the citrate sensitivity of the enzyme whereas dephosphorylation of some other sites leads to overall (citrate-dependent and independent) increase in the activity (20). Some of the phosphorylation sites are related to activity whereas others are silent sites. To determine if altered citrate sensitivity of ACC with age is related to phosphorylation status, we measured phosphate content of purified enzyme. The purified ACC preparations from different age groups had a similar number of phosphate groups. This suggests that the age-associated changes in ACC activity may not be related to changes in the overall phosphorylation state of the enzyme. The increased citrate sensitivity of ACC from 12-month-old rats may be due to altered rearrangement of phosphate groups on this enzyme such that the occupancy of critical phosphorylation sites favors increased citrate sensitivity. The analysis of these

specific ACC phosphorylation site rearrangements is technically very challenging and is beyond the scope of the current study.

The observed decrease in ACC activity in hepatic homogenates of 24-month-old rats compared with 12-month-old rats paralleled the decrease in hepatic ACC mRNA levels (Fig. 4). This indicates that the changes in ACC during senescence occur at least partly at a pretranslational level. On the other hand, the increase in ACC activity in total hepatic homogenates or purified enzyme from 12-month-old rats compared with 4-month-old rats must have been secondary to stoichiometric changes in ACC.

A potential confounding variable is that ACC expression is dependent on nutritional intake, and the changes observed in 24-month-old rats may have been related partially to reduced food consumption relative to young rats. Reduced food intake is known to downregulate ACC activity whereas high-carbohydrate, low-fat diet stimulates ACC mRNA and activity level (21).

Aging in rats is associated with a host of changes in various lipogenic enzymes such as malic enzyme, fatty acid synthase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase (7–9). However, most of the changes are maturational and in the postpubertal rats often occur very modestly over time. The changes in ACC reported in this study, namely an increase in activity at 12 months and decreased activity at 24 months, correlate with the known changes in body content of adipose tissue in this strain of rats. Body fat mass is significantly increased in 12-month-old rats, and subsequently in late senescence some loss of body fat mass is observed (6). The precise cause of this anorexia and cachexia associated with advanced age is not known.

The present study suggests that changes in ACC may be a key biochemical pathway in lipogenesis in aging rats. These changes occur both at a pretranslational and post-translational level. The post-translational changes could not be ascribed to alterations in the total phosphate content of the enzyme. However, alterations in the occupancy of selective phosphorylation sites can not be excluded.

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