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Acetyl-CoA Carboxylase in Reuber Hepatoma Cells: Variation in Enzyme Activity, Insulin Regulation, and Cellular Lipid Content

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Abstract Reuber hepatoma cells are useful cultured lines for the study of insulin action, lipid and lipoprotein metabolism, and the regulation of acetyl-CoA carboxylase (ACC), the rate-limiting enzyme of fatty acid biosynthesis. During investigations in different clonal lines of these cells, we have uncovered marked intercellular variability in the activity, enzyme content, and insulin regulation of ACC paralleled by differences in cellular neutral lipid (triglyceride) content. Two contrasting clonal lines, Fao and H356A-1, have been studied in detail. Several features distinguish these two lines, including differences in ACC activity and enzyme kinetics, the content of the two major hepatic ACC isozymes (M_r 280,000 and 265,000 Da) and their heteroisozymic complex, the extent of ACC phosphorylation, and the ability of ACC to be activated on stimulation by insulin and insulinomimetic agonists. As studied by Nile Red staining and fluorescence-activated cell sorting, these two lines also display marked differences in neutral lipid content, which correlates with both basal levels of ACC activity and inhibition of ACC by the fatty acid analog, 5-(tetradecyloxy)-2-furoic acid (TOFA). These results emphasize the importance of characterization of any particular clonal line of Reuber cells for studies of enzyme regulation, substrate metabolism, and hormone action. With respect to ACC, studies in contrasting clonal lines of Reuber cells could provide valuable clues to understanding both the complex mechanisms of intracellular ACC regulation in the absence and presence of hormones and its regulatory role(s) in overall hepatic lipid metabolism.

Key words: Reuber hepatoma, protein phosphorylation, Nile Red, triglycerides, fatty acid

Reuber hepatoma cells, derived from a chemically induced rat hepatoma [1], have been widely employed as cultured cell lines for the study of hepatic metabolism. Several different clonal lines have been established that differ in several ways, including growth characteristics and the presence or absence of liver-specific proteins [2–5]. Certain clonal lines, like Fao, have been employed by many investigators in the study of insulin action, because of their extreme sensitivity to insulin and the ability of insulin to exert both rapid and long-term effects [6–8]. This same line has also been reported to be a good model for the study of lipoprotein synthesis and secretion [9,10]. We have previously reported on the use of the Fao line in the study of insulin regulation of the rate-limiting extramitochondrial enzyme of fatty acid synthesis, acetyl-CoA carboxylase (E.C. 6.4.1.2; ACC) [11]. As these studies were extended to other clonal lines of Reuber cells, it became apparent that different lines display marked variation in ACC activity and its ability to be regulated acutely by insulin. In this report, we have studied these variable features in two different clonal lines, Fao and H356A-1, in order both to characterize in detail the nature of the ACC activity differences and variable insulin responsiveness and to correlate variable activity with cellular neutral lipid content. These data, though limited to the study of ACC, are presented to emphasize the importance of characterization of any particular clonal line of Reuber cells for studies of enzyme regulation, substrate metabolism, and hormone action.

Abbreviations used: ACAT, acyl-CoA: choleterol acyltransferase; ACC, acetyl-CoA carboxylase; ELISA, enzyme-linked immunosorbent assay; TOFA, 5-(tetradecyloxy)-2-furoic acid.

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MATERIALS AND METHODS

Materials

NaH[^{14}C]O_3 and [^{32}P]-P_i (carrier-free) were obtained from ICN. [^{125}I]-labeled goat anti-mouse Ig was purchased from Dupont/New England Nuclear; [^{125}I]-goat anti-rabbit Ig and [^{125}I]-Protein A were purchased from ICN. Alkaline phosphatase-linked goat anti-rabbit or anti-mouse Ig was purchased from Southern Biotechnology. Cell culture media were obtained from Gibco/BRL and calf serum from Hazelton. Male Sprague-Dawley rats (130–150 g) were purchased from the Charles River Breeding Laboratories. Fao cells were obtained from Dr. C. Ronald Kahn (Harvard) and H356A-1 cells from Dr. Nobuyoshi Shimizu (Arizona). Porcine crystalline insulin was a kind gift of Dr. Ronald Chance (Lilly). A polyclonal anti-phosphotyrosine antibody was obtained from Dr. Gustav Lienhard (Dartmouth). TOFA was obtained from the Merrell Dow Research Institute (Cincinnati, Ohio). Nile Red and the ACAT inhibitor were the kind gifts of Dr. T.Y. Chang (Dartmouth). All other chemicals were obtained from Sigma.

Methods

Cell culture. Fao and H356A-1 hepatoma cells were grown in RPMI 1640 supplemented with 5% fetal calf serum, 5% calf serum and penicillin/streptomycin. Cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37° to subconfluence (60–70%) in the presence of serum in 10-cm plastic dishes (Corning). All of the reported studies were then carried out under serum-free conditions. For agonist stimulation experiments, cells were washed three times with phosphate-buffered saline (PBS) followed by a 1 hour incubation in serum-free medium; this cycle of washing and incubation was then repeated two additional times prior to agonist exposure and cell lysis. Identical results were obtained after overnight serum-free incubation (not shown); the shorter period of serum-free culture was employed for convenience. For ^{32}P-labeling, after the cycle of serum-free incubation, the cells were then cultured in P_i-free RPMI 1640 containing 0.2 mCi carrier-free $^{32}P_i$ per ml for an additional 2 hours.

Cell lysate preparation for ACC assay and isolation. Cell lysates were prepared after washing the cells with PBS by digitonin lysis into a buffer (0.4 ml per 10 cm plate) containing Tris-Cl (50 mM; pH 7.5), EDTA (1 mM), NaF (0.1 M), β-mercaptoethanol (10 mM), sucrose (0.25 M), digitonin (0.4 mg/ml), and seven protease inhibitors [11]. At this digitonin concentration, all ACC was shown to be released by digitonin from these cell lines, as determined by immunoblotting of lysates, residual sonicated cells after digitonin lysis, and total cell sonicates (not shown). With lysates prepared for enzyme isolation, the lysate was then immediately added to 0.1 vol of the same buffer containing 60 mg/ml fatty acid-free bovine serum albumin. ACC was then isolated on 1-ml monomeric avidin-Sepharose columns [11,12].

ACC activity and content measurement. ACC activity in lysates and isolates was determined by H$^{14}CO_3$-fixation to acid-stable products at varying citrate or acetyl-CoA concentrations [as in 11,13]. Enzyme kinetics were analyzed with the Eadie-Hofstee transformation; parameters of linear kinetics were determined using Cricket Graph 1.2, while non-linear kinetics were analyzed by piecewise linear regression analysis (NONLIN program of SYSTAT) with a Macintosh IIcx computer [14,15].

ACC mass and isozyme distribution in lysates and isolates was determined by immunoblotting and/or by an avidin-based sandwich ELISA with 3 different antibodies [as in 16,17]. In order to express data as μg of isozyme, varying loads of purified rat liver enzyme were analyzed on the same immunoblot or ELISA. For immunoblotting of both isozyme species (analyzed as individual bands), the standard curve was linear between 10 and 100 ng of total ACC protein, while the ELISA assay is linear between 0 and 6–9 ng of total ACC. Since the individual liver isozymes have not yet been separated in a undenatured form, the following assumption was made in order to calculate individual isozyme mass. Since total ACC isolated from rat liver is comprised of approximately 90% ACC 265 and 10% ACC 280 on Coomassie staining of SDS gels [16], this ratio was employed to estimate the individual masses in the standard curve. For example, 100 ng of total ACC (measured by the Coomassie Blue assay of Bradford [18]) was taken to be 90 ng of ACC 265 and 10 ng of ACC 280. It was further assumed that isozyme reactivity in cellular enzyme was similar to that of whole liver and that there was no quantitative difference in im-

munoreactivity in individual isozymes between cell lines.

The mass of the heteroisozyme complex between ACC 265 and ACC 280 was measured by a double antibody sandwich ELISA [16,17]. Equal amounts of lysate protein (150–200 μg) were assayed in each ELISA. The assay time (after p-nitrophenyl phosphate addition) was 15 min, within a linear range, as determined by simultaneous analysis of varying loads of fasted/refed liver enzyme (50–500 ng). Results are expressed as A_{415} per mg lysate protein.

Nile Red staining and fluorescence-activated cell sorting (FACS). After 24 hours of serum-free incubation (with or without the addition of either an acyl-CoA: choleterol acyltransferase (ACAT) inhibitor [19] or 5-(tetradecyloxy)-2-furoic acid (TOFA) [20] [see text], cells were washed rapidly three times with ice-cold phosphate-buffered saline (PBS) and harvested by trypsinization, which was terminated after 5 min by the addition of cold medium containing 10% calf serum. The cells were pelleted at 1,000*g* for one min, washed three times with ice-cold PBS, resuspended in PBS (3 ml per dish), and transferred to plastic capped tubes. Cells were then stained with Nile Red, a fluorescent dye, which partitions into intracellular neutral lipid droplets [21]. Thirty microliters of a Nile Red stock solution (10 μg/ml in ethanol) were added to the cell suspension, which was mixed gently and allowed to sit at room temperature in the absence of light for 15 min. Control unstained cells for determination of endogeneous fluorescence on FACS analysis were treated identically except for incubation with ethanol vehicle alone. Cells were analyzed [as in 22] on an Ortho System 50H Cytofluorograph with a Coherent argon-ion laser interfaced with a 2150 Computer system for automatic background subtraction and data processing. Approximately 10^5 cells were injected for each run. A linear scale reading the 90° green signal at 525 nM (300 mwatts) after excitation at 488 nM was employed. Data output from these analyses is presented as mean fluorescence intensity (arbitrary units) as well as a graphic representation of fluorescence intensity versus cell size. Parallel cell plates incubated identically for the 24 hour period were harvested by digitonin lysis, as above, for estimates of ACC activity and the activities of fatty acid synthase and ATP-citrate lyase [23]. Fluorescence microscopy and photography was performed [as in 22]. TOFA was prepared as a 1 mM stock solution for these incubations by drying an acetone solution under a N_2 stream followed by stirring the residue for 1 hour in serum-free medium containing fatty-acid-free bovine serum albumin (BSA; 2.5% [w/v]). The ACAT inhibitor was dissolved in dimethylsulfoxide (0.2 mg/ml) and directly added to cell medium at a 1:4,000 dilution.

Total lipid synthesis from acetate and oleate. In concert with measurement of the effects of TOFA on cell fluorescence and ACC activity, serum-free cells incubated in the presence or absence of TOFA for 24 hours were then also incubated in replicate for 2 hours in medium containing either ^{14}C-acetate (5 mM; 0.5 μCi/μmol) or ^{14}C-oleate (0.1 mM; 2.1 μCi/μmol). Cells and medium were then extracted with chloroform:methanol (2:1) followed by separation of cellular lipids on thin layer chromatography (TLC) (petroleum ether:ethyl ether:acetic acid 80:20:1). Areas corresponding to stained TLC standards for phospholipid, triglyceride, free cholesterol, cholesterol ester, and fatty acid were scraped into scintillation vials and ^{14}C content measured in a toluene-based scintillation fluor (Packard Instruments). In parallel plates, cells were harvested in 1 N NaOH for the determination of protein content per plate.

Other methods. ACC for use in kinase assays and as an immunoblotting standard was isolated from the livers of fasted/refed rats by monomeric avidin-Sepharose chromatography [as in 12]. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [24]. DNA synthesis, tyrosine aminotransferase activity, and aminoisobutyric acid uptake were measured [as in 25]. Immunoblotting of phosphotyrosine-containing proteins was performed [as in 26]. The insulinomimetic oligosaccharide was partially purified from the medium of serum-starved H-35 hepatoma cells by extraction, anion-exchange chromatography, and P2 Biogel chromatography [as in 25].

RESULTS

ACC Activity, Mass, and Isozyme Composition in Cell Lysates

Basal ACC activity is markedly different between Fao and H356A-1 cells. Total ACC activity, expressed as mU per mg lysate protein, is higher than that in the Fao line, whether measured in the absence or presence of saturating

TABLE I. ACC Activity and Content in Fao and H356-1 Cell Lysates†

	Fao	H356A-1
ACC, mU/mg lysate protein		
Citrate 0 mM	0.27 ± 0.06*	0.55 ± 0.05
Citrate 10 mM	3.20 ± 0.26*	4.21 ± 0.12
ACC, μg/mg lysate protein		
ACC 265	1.22 ± 0.20	1.33 ± 0.10
ACC 280	0.07 ± 0.005*	0.04 ± 0.003
Total ACC	1.29 ± 0.21	1.38 ± 0.09
Lysate protein, μg/10^6 cells	27.8 ± 2.5	37.0 ± 2.3*
Total ACC content (ng/10^6 cells)	47.6	38.4
ACC, U/mg total ACC protein		
Citrate 0 mM	0.21 ± 0.04*	0.40 ± 0.04
Citrate 10 mM	2.49 ± 0.20*	3.05 ± 0.09

†ACC activity at 0 and 10 mM citrate and isozyme content (means ± SD) were determined in lysates derived from six separate experiments with paired platings of Fao and H356A-1 cells. All enzyme assays were conducted in duplicate and immunoblotting analyses of lysate proteins for ACC 280 and 265 content performed in triplicate with the sheep polyclonal antibody that recognizes both ACC isozymes. Mass of isozymes was calculated as in Methods; total ACC mass (ACC 265 + ACC 280) was used to calculate actual specific activity. In a separate plating of cells (n = 6 each line), cell lysate protein and cell number per plate were compared in order to estimate ACC content per cell.
*$P < 0.001$ (or less) Fao vs. H356A-1.

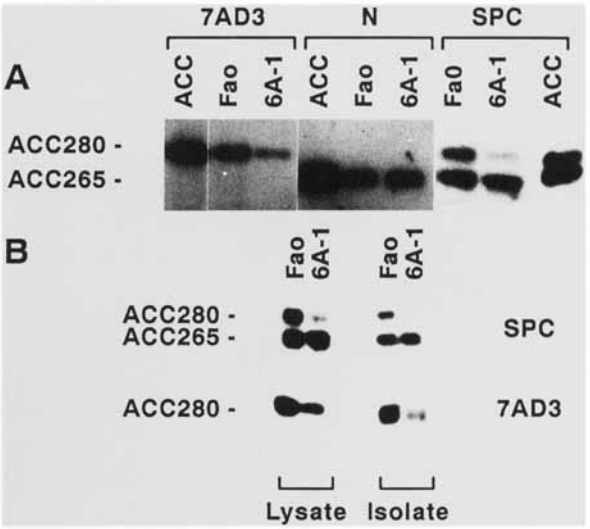

Fig. 1. Immunoblotting of Fao and H356A-1 ACC isozymes in lysates and isolates. Shown is a montage of representative immunoblots of cell lysates and ACC isolates from Fao and H356A-1 (6A-1) cells after separation of proteins on 5% acrylamide gels and transfer to nitrocellulose. **A:** Aligned immunoblots of 50 μg of lysate protein probed with the monoclonal specific for ACC 280 (7AD3), an anti-N-terminal anti-peptide antibody (N) specific for ACC 265, and a sheep polyclonal (SPC) antibody that recognizes both isozymes. Within each set is also shown the blot of 40 ng of fasted/refed liver ACC for comparison. The relative content of ACC 265 was also confirmed with two additional antibodies, an anti-C-terminal antipeptide antibody and a rabbit polyclonal antibody raised against ACC 265 [16] (data not shown). **B:** Immunoblots of cellular lysates (50 μg total protein) and ACC isolates (avidin-Sepharose chromatography; 16 μl of pooled isolate) from the 2 cell lines probed with either the ACC 280–specific antibody (7AD3) or the sheep polyclonal antibody (SPC). Only portions of each immunoblot are shown; no immunoreactivity was noted with any of these antibodies on the remainder of the blot.

citrate (10 mM) (Table I). This higher basal activity of ACC in the H356A-1 cell lysates could be due to an increase in enzyme content, an increase in enzyme specific activity, or different isozyme composition. Immunoblotting with isozyme-specific antibodies demonstrates that the two major hepatic ACC isozymes (molecular mass 280,000 Da [ACC 280] and 265,000 Da [ACC 265]) are present in each cell line, although the mass of the former is markedly lower in H356A-1 cells (Fig. 1A). Estimation of the content of each isozyme by quantitative immunoblotting (confirmed by ELISA) reveals that the mass of ACC 265 is identical in Fao and H356A-1 lysates (normalized for lysate protein), while ACC 280 content in the H356A-1 lysate is 57% of that in Fao cells (Table I). There is a greater amount of protein (as μg/10^6 cells lysed) released from Fao cells by digitonin; therefore, the total mass of both digitonin-releasable ACC specific per cell is about 24% lower in the H356A-1 line. The calculated actual ACC specific activity (as U/mg of ACC protein) in these lysates (assuming that the total mass of ACC is the sum of the isozyme masses and assuming equal specific activities of each[1]) is about twofold higher in the H356A-1 lysates in the absence of citrate and 32% higher at maximal velocity (10 mM citrate).

[1]Since they have not been independently isolated from rat liver or hepatoma cells, it is possible that the maximal velocities of the individual isozymes are indeed not identical (V_{max}/mg enzyme). Obviously, this could vary the estimation of the total ACC specific activity; since only the content of ACC 280 (the minor species in whole liver enzyme) is different between the cells (per mg lysate protein), this would have only a small effect on this estimate of total specific activity, unless the differences in specific activities were very large. Whether the data are expressed in this fashion or not corrected for individual enzyme mass in the lysates, the major point is that the higher ACC activity in H356A-1 cell lysates cannot be accounted for by a greater ACC mass of either isozyme.

ACC Isolation From Fao and H356A-1 Cells: Activity and Kinetics

The higher specific activity of total ACC in H356A-1 lysates could be due to the effects of allosteric regulators present in the lysates. If allosteric regulators of ACC, such as citrate or fatty acyl-CoA, were responsible for the observed activity differences and not covalent modification or other structural differences, then the activity of the purified enzyme from each line should be identical, since these ligands would be removed during purification. Accordingly, ACC was isolated to homogeneity from Fao and H356A-1 cells by monomeric avidin-Sepharose chromatography. The difference in ACC specific activity (as U/mg ACC protein) between Fao and H356A-1 enzyme, however, persists to the same extent following enzyme isolation (Table II, columns 6 and 7; representative of 4–6 isolations from unlabeled and labeled cells). The estimated maximal enzyme velocities (U/mg ACC at saturating citrate) of the isolated enzymes are somewhat lower than that predicted from measurement of enzyme activity and content in the crude lysates, with a greater difference between the two cell lines (compare Tables I and II). There is also a relative increase in fractional activity measured in the absence of citrate. In other experiments (not shown), we have noted that the proportion of the enzyme active in the absence of citrate reproducibly increases on purification. This increase in citrate-independent activity is noted after the dialysis step prior to avidin-Sepharose chromatography, suggesting removal of a small ligand that inhibits citrate-independent activity in the lysates.

The K_a for citrate of the purified H356A-1 enzyme, determined from linear Eadie-Hofstee plots of enzyme velocities at variable citrate concentrations, is lower than that for the purified Fao enzyme (Fao isolates: 0.64 ± .06 mM; H356A-1 isolates: 0.33 ± .08 mM [means ± SD of four isolates from each cell type]). The two isolated enzymes also show differences in acetyl-CoA kinetics. Unstimulated cells display linear acetyl-CoA kinetics on Eadie-Hofstee plots with a higher AcCoA K_m in the H356A-1 isolates (Fao: 44 ± 5 (SD) μM; H356A-1: 78 ± 9 μM [analysis of 6 Fao and 3 H356A-1 isolates]) (Fig. 2A,B). The isozyme distribution in the isolates is identical to that observed in the crude lysate, indicating equal recovery of each individual isozyme (Fig. 1B).

Taken together, these data argue strongly that structural differences in either isozyme polypeptide, the varying isozyme composition, or differential post-translation modification (e.g., phosphorylation) of total ACC accounts for the differences in ACC activity between these two cell lines.

^{32}P-ACC Isolation From Fao and H356A-1 Cells

Differences in citrate reactivity are a function of the state of phosphorylation, the dephospho-

TABLE II. Comparison of ^{32}P Content and Enzyme Activity in ACC Isolated From ^{32}P-Labeled H356A-1 and Fao Cells*

	V_5 (mU/ml)	V_0 (mU/ml)	^{32}P (cpm/ml)	Mass ACC 265 (μg/ml)	Mass ACC 280 (μg/ml)	ACC SA V_5 (U/mg ACC)	ACC SA V_0 (U/mg ACC)	^{32}P Content (cpm/μg)	K_a Citrate (mM)
H356A-1									
Isolate 1	2.43	0.55	2439	1.10	.030	2.15	0.49	2217	0.39
Isolate 2	2.43	0.31	1944	1.03	.032	2.29	0.29	1887	0.40
Fao									
Isolate 1	1.31	0.15	8559	0.99	.061	1.25	0.14	8645	0.69
Isolate 2	1.69	0.19	7071	1.06	.057	1.51	0.17	6671	0.59

*^{32}P-ACC was isolated from labeled Fao and H356A-1 cells, as in Methods. Shown are the data from two parallel independent isolates from each line derived from separate platings of cells. ACC activity in the isolates (expressed as mU/ml) was measured at variable citrate concentrations; shown are data from assays performed in the absence of citrate (V_0) and at a maximally stimulating citrate concentration (5 mM; V_5). The K_a for citrate was determined from linear Eadie-Hofstee plots (analyzed by Cricket Graph 1.2) of V vs. V/[Citrate (mM)] (where V equals enzyme velocity after subtraction of citrate-independent activity) for assays conducted at variable citrate concentrations between 0.1 and 5 mM. ^{32}P content (cpm/ml) was determined by Cerenkov counting of the dialysed isolates used for enzyme assay. Enzyme mass in isolates was determined by immunoblotting with a sheep polyclonal antibody that recognizes both isozymes, as in Methods. Enzyme specific activity (SA) is expressed as units per mg total ACC (ACC 265 + ACC 280). Since no phosphorylation of ACC 280 could be detected in these experiments with all recognizable radioactivity migrating with the ACC 265, ^{32}P content is expressed as cpm/μg of ACC 265.

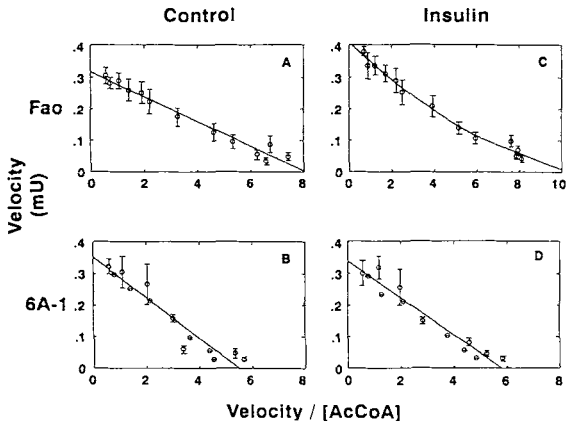

Fig. 2. Acetyl-CoA kinetics of ACC isolates from Fao and H356A-1 cells. Shown are Eadie-Hofstee plots (velocity (mU) vs. velocity/[acetyl-CoA]) of data derived from the activities of ACC isolates, measured at variable acetyl-CoA concentrations in the presence of 10 mM citrate. Isolates were prepared by avidin-Sepharose chromatography from control and insulin-stimulated (10 nM; 30 min) Fao (n = 6) and H356A-1 (n = 3) cells, as described in Methods (**A:** control Fao; **B:** control H356A-1 (6A-1); **C:** insulin Fao; **D:** insulin H356A-1). The data are displayed as means (±SD) of actual experimental points; the line in each instance was fitted by regression analysis (linear or non-linear) using the MGLH or NONLIN programs in SYSTAT, respectively [14,15].

rylated enzyme possessing higher citrate independent activity, lower K_a for citrate, and an increase in V_{max} (saturating citrate) [11,27,28]. The activity differences between the two enzymes thus suggested that ACC might be relatively dephosphorylated in the H356A-1 cells as compared to Fao cells. This prediction was tested by isolating ^{32}P-ACC from each cell line after labeling in intact cells under serum-free conditions. After identical conditions of cell labeling with $^{32}P_i$ and enzyme isolation, the H356A-1 enzyme is, indeed, dephosphorylated relative to the Fao enzyme, coincident with differences in specific activity, citrate dependence, and isozyme distribution (Table II). The change in phosphorylation state was only recognized in ACC 265 on radioautography in these analyses (not shown). We were not able to detect ^{32}P-phosphate incorporation into ACC 280 in these experiments; it remains to be established whether in fact ACC 280 is a phosphoprotein in these cells or whether it is extensively dephosphorylated in both cell lines.

ACC Activation by Insulin

In serum-deprived Fao cells, insulin acutely increases the activity of ACC, as measured in cellular lysates. As previously reported [11], this effect is largest in the measurement of citrate-independent activity (2–2.5-fold), but there is also an increase in activity in the presence of a maximally saturating citrate concentration. (Fig. 3, left-hand panels). Maximal activation of ACC in the Fao line is observed at about 10 nM insulin with half-maximal activation at about 5×10^{-10} M (not shown). However, in H356A-1 cells, no effect of insulin (at concentrations up to 1 μM) is evident following assay of ACC at either citrate concentration (Fig. 3, right-hand panels). Insulin also alters the acetyl-CoA kinetics of ACC in Fao cells, resulting in a curvilinear Eadie-Hofstee plot; this response is absent in the H356A-1 cells (Fig. 2, right-hand panels). This overall non-responsiveness of ACC to insulin was also observed in other Reuber cell lines (H35 (the parental line for the H356A-1), H4IIEC3, and H4IIE) (not shown).

We have previously demonstrated by immunoprecipitation and sandwich ELISA that there exists a heteroisozyme complex between ACC 265 and ACC 280 in preparations of isolated ACC from liver, mammary gland, and brown adipose tissue (where both isozymes are present) [16,17]. Such complexes are also detectable in control lysates from both Fao and H356A-1 cells

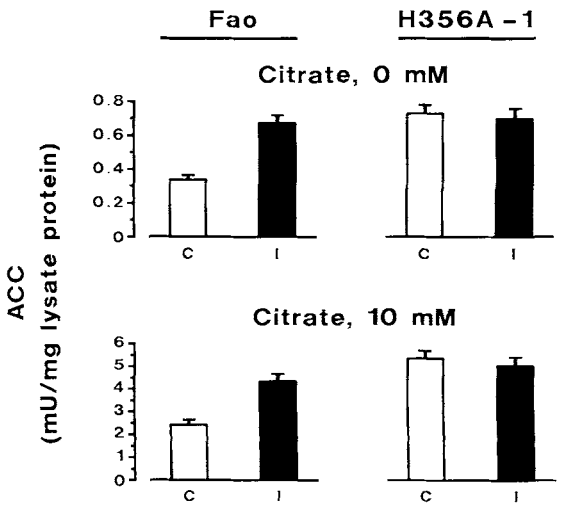

Fig. 3. Effect of insulin on ACC activity in cell lysates from Fao and H356A-1 cells. Shown are results obtained from four separate paired experiments in which ACC activity was determined at 0 (upper panels) and 10 mM (lower panels) citrate in cell lysates from Fao (left-hand panels) and H356A-1 (right-hand panels) cells after a 30 min exposure to either control vehicle (C, open bars) or insulin (10 nM; I, solid bars). The data are represented as the means (±SD) of activity expressed as mU per mg lysate protein.

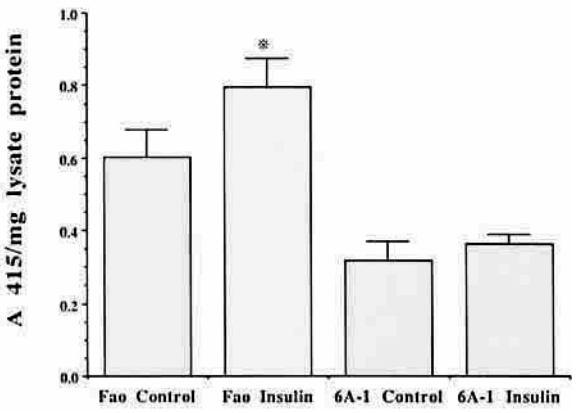

Fig. 4. Mass of the ACC heteroisozyme complex in Fao and H356A-1 cells before and after insulin stimulation. The mass of the heteroisozyme complex between ACC 265 and ACC 280 was measured in Fao (n = 3) and H356A-1 (n = 3) lysates by double antibody sandwich ELISA, as in Methods. Lysates were prepared after a 30 min exposure to control vehicle or insulin (10 nM) prior to cell disruption. Experiments were performed with parallel platings of the cell lines in each instance; equal amounts of lysate protein (150–200 μg) were assayed in each experiment. The assay time (after p-nitrophenyl phosphate addition) was 15 min, within a linear range, as determined by simultaneous analysis of varying loads of fasted/refed liver enzymes (50–500 ng). Results are expressed as A_{415} per mg lysate protein (±SE). * indicates $P < 0.05$ control vs. insulin.

by ELISA analysis (Fig. 4); the amount of complex is 47% lower in H356A-1 lysates (a degree commensurate with a lower mass of ACC 280). This difference persists after enzyme isolation from unstimulated cells of both lines. The amount of detectable isozyme complex increases by a small but significant degree in Fao lysates after insulin stimulation (and insulinomimetics [not shown]) (Fig. 4). However, no increase in complex mass in response to insulin (or other agents) is seen in the H356A-1 lysates.

The non-responsiveness to insulin in the H356A-1 cells is restricted to ACC activation in the present analysis. In these cells, insulin is able to stimulate DNA synthesis, induce tyrosine aminotransferase, and stimulate amino acid uptake to the same extent as in the Fao line (not shown). We have also noted that insulin stimulates the tyrosine phosphorylation of a cytosolic protein of M_r = 180,000 in these cells in a manner indistinguishable from Fao cells (not shown). The "ACC activation resistance" is, however, not restricted to insulin in H356A-1 cells. ACC in this cell line cannot be activated by three other insulinomimetic agonists, as compared to Fao cells. Neither orthovanadate, an insulinomimetic oligosaccharide [25], or calf serum is able to activate ACC, yet all are active on the Fao line (not shown). These data suggest strongly that the insulin receptor is fully competent for signalling and that the "resistance" of ACC to activation in the H356A-1 cells must be due to another mechanism.

Correlation of Cell Neutral Lipid Content With Basal ACC Activity

Because ACC is the rate-limiting step in fatty-acid synthesis, its activity could also be reflected in the rate/extent of synthesis of fatty acid esters, such as triglyceride and phospholipid. Inhibition of ACC activity in rat hepatocytes markedly diminishes the rates of production of these lipid species [20,29]. Thus, it was of interest to compare neutral lipid content in these two cell lines and to attempt to relate ACC activity to accumulation of these lipid species. Nile Red is a fluorescent dye which stains for neutral lipid and has been extensively employed in the study of intracellular cholesterol ester metabolism [21,22]. As observed on fluorescence microscopy, there is a striking difference in the Nile Red O fluorescence between these two cell lines with the heavily staining H356A-1 cells displaying larger and more numerous lipid droplets (Fig. 5). This heightened fluorescence in the H356A-1 cells, when quantified by fluorescence-activated cell sorting (Fig. 6; compare panels A and D), is not diminished after incubation of cells with an inhibitor of acyl-CoA: cholesterol acyl transferase (ACAT [19]) (Fig. 6; compare panels D and E), indicating that differences in cholesterol ester content do not account for differences in cell fluorescence. However, prior incubation of H356A-1 cells with an inhibitor of ACC, 5-(tetradecyloxy)-2-furoic acid (TOFA [20]), markedly diminishes cellular fluorescence to the staining profile observed in Fao cells (Fig. 6; compare panels C and F). Determination of mean cell fluorescence by this technique was highly reproducible, as shown by staining and FACS analysis of triplicate culture dishes in the experiment shown in Figure 6 (FAO cells: Control, 209 ± 26, ACAT inhibitor, 358 ± 54, TOFA, 234 ± 7; H356A-1 cells: Control, 598 ± 50, ACAT inhibitor, 682 ± 97, TOFA, 198 ± 10 arbitrary fluorescence units ± standard deviation). ACC activity, in lysates derived from parallel cell platings, is also inhibited by TOFA in the H356A-1 cells to the level of activity in the Fao line, while the ACAT inhibitor has little or no effect on ACC activity (Fig. 6 inserts). The cellular content of both ACC isozymes, assessed

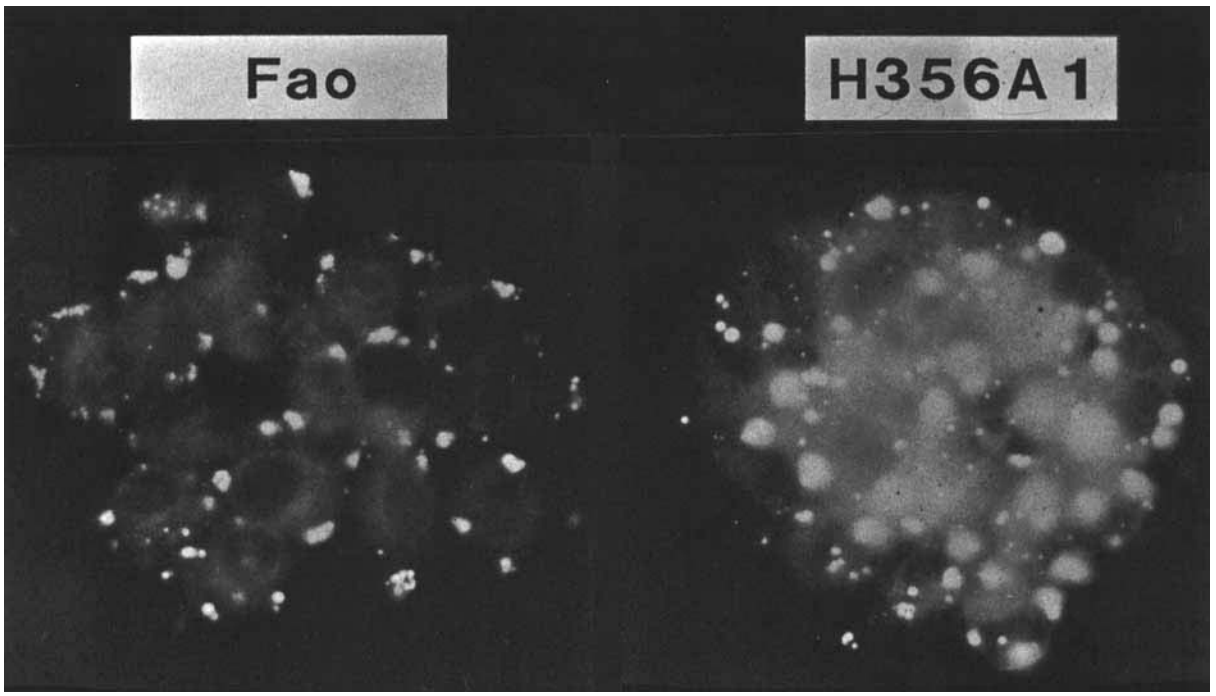

Fig. 5. Nile Red staining of Fao and H356A-1 cells. Cells, grown on glass coverslips, were stained in situ with Nile Red after 24 hours of serum-free incubation and examined with a Zeiss fluorescence microscope (excitation 485 nM; emission 520–560 nM) (as in [22]). Representative fields of cells from each line, photographed at identical microscope and camera settings, are shown.

by immunoblotting, is unaltered by TOFA exposure over the time period of incubation (not shown), indicating that TOFA causes exclusively a change in ACC specific activity.

The inhibition of ACC activity by TOFA in the H356A-1 cells is accompanied by marked decreases in the synthesis of neutral lipid (triglyceride and sterol ester) and phospholipid from ^{14}C-acetate, while lipid esterification from ^{14}C-oleate is largely unaffected (Fig. 7A,B). As shown in Fig 7C, there is also a parallel concentration-dependent inhibition of both mean cellular fluorescence intensity and ACC activity by TOFA with an I_{50} for each of approximately 2.5 μM. The activities of ATP-citrate lyase and fatty acid synthase in cellular lysates were not significantly changed over this TOFA concentration range in this same experiment (not shown). Taken together, all of these data are consistent with a selective inhibition of ACC accounting for the TOFA-induced changes in mean cellular fluorescence intensity.

DISCUSSION

These studies were undertaken after the discovery of marked variations in the basal activity of acetyl-CoA carboxylase and its ability to be activated in response to insulin in several different clonal lines of Reuber hepatoma cells. We elected to compare in some detail the Fao line and H356A-1 line in this report in an attempt to understand the mechanisms underlying this variability and their impact on the cellular phenotype. Since the detailed mechanism by which insulin acutely regulates ACC activity remains unknown, the delineation of these interesting differences between clonal lines of the same progenitor cell (H35 [2]) might provide some insight into some of these regulatory mechanisms.

Variation in Basal ACC Activity

Several features distinguish the total ACC in the H356A-1 cells from that in the Fao line in unstimulated cells. These include a lower digitonin-releasable total content per cell, a decrease in the M_r 280,000 isozyme content and the mass of the heteoisozyme complex, higher basal specific activity, a lower K_a for citrate, a higher K_m for acetyl-CoA, and relative dephosphorylation of the M_r 265,000 isozyme. Because the differences in ACC activity and kinetics persist through purification of the enzyme to homogeneity, intercellular variations in enzyme structure,

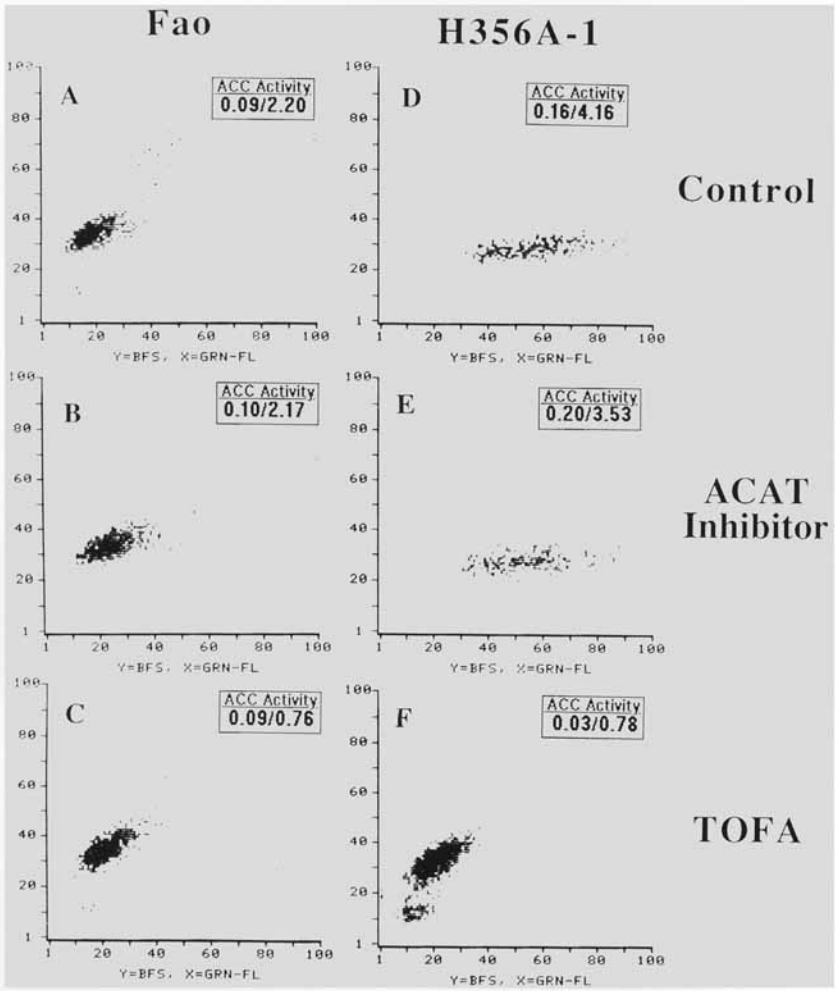

Fig. 6. Effect of TOFA and ACAT inhibitor on cell fluorescence and ACC activity. Shown are the original unretouched FACS profiles (10^5 cells injected in each) of Nile Red stained Fao cells (left-hand panels) and H356A-1 cells (right-hand panels) after treatment for 24 hours in serum-free medium with the ACAT inhibitor (50 ng/ml; **B, E**), TOFA (20 μM; **C, F**), or control addition (**A, D**). Within each panel is also shown the ACC activity measured at 0 and 10 mM citrate in lysates derived from parallel incubations (as mU/mg lysate protein). The abscissa in each panel represents green fluorescence intensity (GRN-FL) in arbitrary units, while the ordinate (BFS or blue forward scatter) equals cell size in arbitrary units.

post-translational modification, or relative isozyme composition are the most likely explanations to account for this basal difference.

First, there could be important differences in the primary structure of either ACC 265, ACC 280, or both. For example, the enzyme could lack important regulatory phosphorylation sites, giving the appearance of relative dephosphorylation on cell labeling with $^{32}P_i$. Since occupancy of these sites influence V_{max} and citrate dependency [27,28,30], their absence in the H356A-1 enzyme(s) might alter these kinetic parameters. In preliminary experiments, however, H356A-1 ACC can be inactivated via apparent phosphorylation by the 5′-AMP-activated ACC kinase in vitro [31]. This suggests that at least some of the multiple regulatory phosphorylation sites are present in the H356A-1 enzyme. Other structural differences not involving post-translational modification could also account for the observed cellular variations (e.g., alterations in acetyl-CoA or citrate binding sites). If any structural differences exist, however, they do not appear to eliminate absolute reactivity with any of our five antibodies, suggesting they may lie in other domains.

Second, although ACC citrate kinetics and the appearance of relative dephosphorylation of the H356A-1 enzyme are internally consistent with differences in enzyme post-translational modifi-

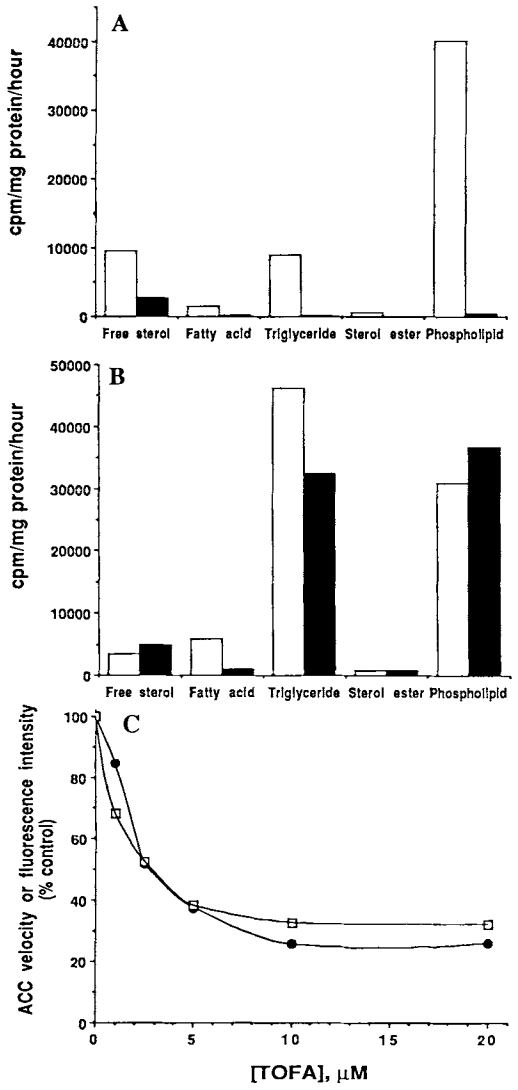

Fig. 7. Effect of TOFA on lipid synthesis, Nile Red fluorescence, and ACC activity in H356A-1 cells. H356A-1 cells, incubated with (shaded bars) or without (open bars) TOFA (20 μM) for 24 hours in serum-free medium, were then pulsed for 2 hours with either ^{14}C-acetate (**A**) or ^{14}C-oleate (**B**), as in Methods. Lipid extracts were separated by thin layer chromatography and ^{14}C-incorporation into various lipid classes (expressed as cpm/mg cell protein/hour) determined. ^{14}C cpm have been corrected in each case for extraction recovery, employing an H-cholesterol internal standard. In **C**, shown is Nile Red fluorescence and ACC activity as a function of TOFA concentration. H356A-1 cells were incubated for 24 hours in serum-free medium with varying concentrations of TOFA (0–20 μM); plates were then processed for Nile Red staining and FACS sorting to determine mean cell fluorescence (●) and for ACC activity measured at 10, 1, and 0 mM citrate (only latter shown) (□). Each sorting and ACC assay was performed on duplicate cell platings. Results are expressed as % 0 TOFA addition (means of 2 determinations).

cation, we cannot as yet ascribe this altered phosphorylation to any detectable differences in the cellular activities of protein kinases or phosphatases active on ACC. In experiments not shown, we have been unable to detect any substantial differences between the cell lines in the activities of the cAMP-dependent protein kinase, the 5′-AMP-activated protein kinase, casein kinase II, or in total ACC protein phosphatase.

Third, while there is a striking difference between the cells in the content of the 280,000 Da ACC isozyme, it is not clear how this might directly relate to differences in total basal ACC activity, since this is the minor species (by mass) of hepatic ACC [16] and its content is substantially lower in the H356A-1 cells that display heightened total activity. The basal diminution in the mass of the complex between the two isozymes can be quantitatively accounted for by the reduction in the mass of the 280,000 Da ACC isozyme. Knowledge of the specific activities of each of the two isozymes would be useful information in understanding the potential impact of changes in relative isozyme composition. However, to date, we have not been able to separate them by either conventional or immunoaffinity chromatography.

Variation in Insulin Responsiveness

The differences in basal ACC activity are also accompanied by the resistance of ACC to insulin activation in the H356A-1 cells. This resistance is expressed in three ways after insulin exposure: absence of a change in citrate reactivity, maintenance of linear acetyl-CoA kinetics, and no change in the mass of the heteroisozyme complex. It cannot be discerned from the present study whether the mechanisms underlying these three phenomena can be explained in a unitary way. While multiple explanations could be envisaged to account for the failure of insulin to affect ACC in these cells, at one level it is perhaps best accounted for by its antecedent heightened basal activity and apparent relative dephosphorylation of the 265,000 Da isozyme. Thus, the basal enzyme in H356A-1 cells has some of the characteristics of the insulin-stimulated enzyme in Fao cells (lower citrate K_a, dephosphorylation of ACC 265) [11]. Since several other insulin responses are intact in the H356A-1 line, it is also possible that unique elements of the ACC activation cascade are altered in these cells. Resistance of ACC to the activating effects of insulin have also been noted in three other Reuber-

derived lines (H35 (the parental line for the H356A-1), H4IIEC3, and H4IIE), although all of the studies completed herein have not been done on each of these lines. These results emphasize that different Reuber-derived cell lines may show striking differences in selected enzymatic systems and their hormonal regulation; care must be taken in reaching conclusions in any study on a single clonal line.

Variation in Neutral Lipid Content

All of these differences in ACC activity and content between the cell lines are accompanied by striking differences in cell neutral lipid (triglyceride) content. The latter could be accounted for either by an excessive rate of neutral lipid synthesis or by diminished lipoprotein secretion or both. The neutral lipid content was estimated after a 24 hour period of serum-free incubation, so that continued synthesis of neutral lipid from medium-derived fatty acid was not contributory (though synthesis antecedent to the time of serum deprivation could have contributed). Most importantly, incubation with the ACC inhibitor TOFA during this period strikingly reduces cellular neutral lipid content, strongly implicating de novo fatty-acid synthesis as a major contributor to the intracellular accumulation. TOFA selectively diminishes accumulation of triglyceride (and phospholipid) synthesized from acetate, but not from long-chain fatty acid, indicating an inhibition of fatty-acid biosynthesis, not fatty-acid esterification. At a qualitative level, these observations suggest a strong link between total ACC activity and the synthesis/content of neutral lipid. While TOFA at the concentrations employed is a reasonably specific inhibitor of ACC [20,29], it has not been exhaustively examined for other possible effects. We recognize that ACC activity and cellular neutral lipid content are, in the extent of the current study, only associated events; there are several other important determinants of the extent of cellular triglyceride accumulation which could differ between Fao and H356A-1 cells.

Nile Red staining coupled with cell sorting has proven to be a useful tool in the study of cholesterol ester metabolism [21,22]. The present results indicate its utility in studying triglyceride metabolism as well. The use of cell sorting coupled with this staining procedure offers the opportunity of selection and subcloning of cells with varying rates of neutral lipid accumulation, either because of enhanced synthesis or diminished export. Given the parallelism in the present study between ACC activity and cellular neutral lipid content, one can also envision its use to potentially select cells with varying ACC activity and regulation. Such clonal lines might display unique properties that could be exploited to delineate many details of the intracellular regulation of ACC and its acute/chronic modulation by nutrients and hormones.

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