Effects of a novel dual lipid synthesis inhibitor and its potential utility in treating dyslipidemia and metabolic syndrome

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Abstract **We have identified a novel ω-hydroxy-alkanedicarboxylic acid, ESP 55016, that favorably alters serum lipid variables in obese female Zucker (fa/fa) rats. ESP 55016 reduced serum non-HDL-cholesterol (non-HDL-C), triglyceride, and nonesterified fatty acid levels while increasing serum HDL-C and β-hydroxybutyrate levels in a dose-dependent manner. ESP 55016 reduced fasting serum insulin and glucose levels while also suppressing weight gain. In primary rat hepatocytes, ESP 55016 increased the oxidation of [^{14}C]palmitate in a dose- and carnitine palmitoyl transferase-I (CPT-I)-dependent manner. Furthermore, in primary rat hepatocytes and in vivo, ESP 55016 inhibited fatty acid and sterol synthesis. The "dual inhibitor" activity of ESP 55016 was unlikely attributable to the activation of the AMP-activated protein kinase (AMPK) pathway because AMPK and acetyl-CoA carboxylase (ACC) phosphorylation states as well as ACC activity were not altered by ESP 55016. Further studies indicated the conversion of ESP 55016 to a CoA derivative in vivo. ESP 55016-CoA markedly inhibited the activity of partially purified ACC. The activity of partially purified HMG-CoA reductase was not altered by the xenobiotic-CoA. These data suggest that ESP 55016-CoA favorably alters lipid metabolism in a model of diabetic dyslipidemia in part by initially inhibiting fatty acid and sterol synthesis plus enhancing the oxidation of fatty acids through the ACC/malonyl-CoA/CPT-I regulatory axis.—** Cramer, C. T., B. Goetz, K. L. M. Hopson, G. J. Fici, R. M. Ackermann, S. C. Brown, C. L. Bisgaier, W. G. Rajeswaran, D. C. Oniciu, and M. E. Pape. **Effects of a novel dual lipid synthesis inhibitor and its potential utility in treating dyslipidemia and metabolic syndrome.** ***J. Lipid Res.*** **2004.** 45: **1289–1301.**

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Systematic chemical modification of long-chain hydrocarbons is one approach to identify novel lipid-regulating compounds for the treatment of human dyslipidemias (1). Over the last 30 years, a variety of compound classes have been identified that demonstrate activity in animal models of dyslipidemia. For example, Parker and coworkers (2) and McCune and Harris (3) have described a series of alkyloxyarylcarboxylic acids, one of which, 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA), has been well studied. TOFA showed marked hypolipidemic activity in both rats and monkeys (2). Another series termed MEDICA (β,β′-methyl-α,ω-dicarboxylic acids) has been developed and extensively studied by Bar-Tana and coworkers (4). In particular, MEDICA 16 has been shown to possess hypolipidemic, anti-diabetic, and anti-atherosclerotic activity in relevant animal models (5, 6). In work spanning many years, Berge, Bremer, and colleagues (7–9) have described a series of 3-thia fatty acids that possess properties similar to those of MEDICA compounds when administered to animal models of dyslipidemia. In addition, Pill and coworkers (10–12) described a series of ω-substituted alkyl carboxylic acids that showed insulin-sensitizing activity and lipid-regulating properties in rodents. Finally, Bisgaier and coworkers (13) described a series of carboxyalkylethers with lipid-regulating activity, including HDL-cholesterol (HDL-C) increase, in rats. One of the carboxyalkylethers, PD-72953, also known as CI-1027 or gemcabene, has been administered to humans and shown to have effects on serum lipid levels (14). Currently, no consensus exists on the primary mechanism of action of long-chain hydrocarbon derivatives that eventually leads to the favorable lipid changes in humans or to the amelioration of metabolic derangements in animal models of dyslipidemia, diabetes, and obesity. Proposed primary mechanisms of action include alterations in enzyme activities through allosteric or redox state changes and modulation of gene expression through the activation or inhibition of nuclear hormone receptors (3, 9, 13, 15–21).

Here, we describe a novel ω-hydroxy-alkanedicarboxylic

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acid, ESP 55016 (**Fig. 1**), that favorably alters serum lipid profiles in the Zucker rat, an animal model of diabetic dyslipidemia. Data from those studies led us to hypothesize that fatty acid oxidation was enhanced by ESP 55016. We have used both in vitro and in vivo models to test this hypothesis while focusing our efforts on identifying the short-term (minutes to hours) metabolic changes induced by ESP 55016 with the goal of identifying the initial primary mechanism(s) of action.

MATERIALS AND METHODS

Materials

DMEM containing 4.5 g/l D-glucose, nonessential amino acids, HEPES buffer, sodium pyruvate, and gentamicin was obtained from Mediatech, Inc. (Herndon, VA). Fetal bovine serum, Dulbecco's phosphate-buffered saline, glutamine, and GlutMax-1™ were obtained from Invitrogen Life Technologies (Grand Island, NY). Bovine albumin fraction V (35% solution), insulin, dexamethasone, [^{14}C]sodium bicarbonate, and zaragosic acid were obtained from Sigma Chemical Co. (St. Louis, MO). The following items were obtained from Perkin-Elmer Life Sciences, Inc. (Boston, MA): [1-^{14}C]acetic acid sodium salt (40–60 mCi/mmol), *R/S*-[2-^{14}C]mevalonolactone (40–60 mCi/mmol), [U-^{14}C] palmitic acid sodium salt (54.5 mCi/mmol), $^{3}H_2O$ (1 mCi/g), [2-^{14}C]pyruvic acid (22 mCi/mmol), and D-[1-^{14}C]glucose (54.5 mCi/mmol). [U-^{14}C]lauric acid (55 mCi/mmol) and [U-^{14}C] caprylic acid (53 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Biocoat® type I collagen-coated six-well plates were purchased from Becton Dickinson Labware (Bedford, MA). ScintiVerse™ scintillation cocktail was obtained from Fisher Scientific Chemicals (Fairlawn, NJ).

Synthesis of ESP 55016

ESP 55016 (8-hydroxy-2,2,14,14-tetramethyl-pentadecanedioic acid), a white crystalline solid, was prepared by a multistep reaction sequence as follows: 1,5-dibromopentane was reacted with ethyl lithio-isobutyrate at low temperature (−40 to −10°C) to produce ethyl 7-bromo-2,2-dimethylheptanoate, which was further used as an alkylating agent in the reaction with *p*-toluenesulfonylmethyl isocyanide (TosMIC). The TosMIC adduct thus obtained was subjected to hydrolysis in acidic conditions to yield 8-oxo-2,2,14,14-tetramethyl-pentadecanedioic acid diethyl ester, which was then reduced at the central keto moiety to a hydroxyl group to afford diethyl 8-hydroxy-2,2,14,14-tetramethyl-pentadecanedioate. Hydrolysis of the ester terminal groups of the latter in basic conditions finally produced the desired compound, ESP 55016.

Obese female Zucker rat study

Ten to 12 week old (400–500 g) obese female Zucker rats [Crl:(Zuc)-faBR] were obtained from Charles River Laboratories and acclimated to the laboratory environment for 7 days. ESP 55016 was dissolved or suspended in a dosing vehicle consisting of 20% ethanol and 80% polyethylene glycol 200 (v/v) or 1.5% (w/v) carboxymethyl cellulose and 0.2% (v/v) Tween 20 (CMC-Tween); we did not observe any significant differences in the responses of compound-treated animals with the two vehicles. The dose volume of ESP 55016 or vehicle was set at 0.25% of body weight. Doses were administered daily by oral gavage for 14 days, approximately between 8 and 10 AM. Animals were fasted for 6 h before blood collection. To measure blood glucose levels, the tail vein was lanced and blood spotted onto a glucometer (Bayer; model 3952E). Before and after 7 days of dosing, a 1.0–2.0 ml sample of blood was collected by administering O_2/CO_2 anesthesia and bleeding from the orbital venous plexus. After 14 days of dosing, blood was collected by cardiac puncture after euthanasia with CO_2. All blood samples were promptly processed for separation of serum and stored at −80°C until analysis.

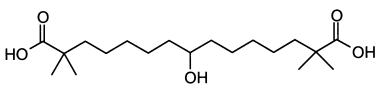

Fig. 1. The structure of ESP 55016.

Serum analysis

Serum triglyceride (Triglycerides Kit 1488872; Boehringer Mannheim Corp., Indianapolis, IN), β-hydroxybutyrate (β-HBA Kit 310A; Sigma Diagnostics, St. Louis, MO), and nonesterified fatty acid (NEFA-C Kit 995-75409; Wako Diagnostics, Richmond, VA) levels were determined with commercially available kits on a Hitachi 912 Automatic Analyzer (Roche Diagnostics Corp., Indianapolis, IN). Serum insulin was determined using an ELISA kit (Alpco Diagnostics, Windham, NH). Serum total cholesterol was assayed by a colorimetric assay based on the method of Allain et al. (22) adapted for the Hitachi 912. Serum lipoproteins were separated by gel-filtration chromatography on a Superose 6HR (1 × 30 cm) column equipped with online detection for total cholesterol as described by Kieft, Bocan, and Krause (23). Total cholesterol content of VLDL, LDL, and HDL lipoproteins were calculated by multiplying the independent values determined for serum total cholesterol by the percentage area of the lipoprotein in the respective profiles.

Hepatocyte isolation

Male Sprague-Dawley [Crl:CD (SD)] rats were fed Purina Certified Rodent Chow pellets (Ralston-Purina Co., St. Louis, MO) and nutritionally staged for hepatocyte isolation by fasting for 48 h (used in studies with [^{14}C]acetate, [^{14}C]pyruvate, [^{14}C]mevalonolactone, $^{3}H_2O$, and ^{14}C-fatty acids) or fasting for 48 h and refeeding a high-carbohydrate diet for 48 h (used in the study with [^{14}C]glucose). Rats were anesthetized with intraperitoneal injection of sodium pentobarbital. Livers were perfused and cells were isolated according the method of Ulrich et al. (24). Hepatocytes were plated in DMEM with glucose and serum (DMEM containing 4.5 g/l D-glucose supplemented to 20% fetal bovine serum, 14 mM HEPES, 0.2% bovine albumin, 2 mM L-glutamine, 1× MEM nonessential amino acids, 100 nM insulin, 100 μg/ml dexamethasone, and 20 μg/ml gentamicin) at a density of 1.5 × 10^5 cells/cm^2 on collagen-coated six-well plastic dishes. After attachment (3–4 h), serum-free medium was used. In all metabolic labeling experiments, radiolabeled compounds and ESP 55016 or vehicle were added to cultures at the same time.

In vitro measurement of mitochondrial β-oxidation

Hepatocyte cultures were incubated in the presence of [^{14}C] palmitic acid, [^{14}C]lauric acid, or [^{14}C]caprylic acid and assayed for the production of acid-soluble products. Experiments were performed using DMEM without D-glucose. All radiolabeled fatty acids were at final concentrations of 200 μM and specific activities of 1 mCi/mmol. Vehicle contained 0.3% DMSO. Fatty acid oxidation rates were measured over 4 h. Incubations were stopped by the addition of perchloric acid followed by extraction with hexane. The acidic aqueous phase was assayed for radioactive content and represented acid-soluble products.

In vitro measurement of lipid synthesis

Hepatocyte cultures were incubated in the presence of [^{14}C] acetate, [^{14}C]pyruvate, [^{14}C]glucose, or $^{3}H_2O$. Experiments were performed in DMEM with glucose plus the appropriate metabolic precursor as follows: 1 μCi/ml [^{14}C]acetate, 0.5 μCi/ml [^{14}C]pyruvate, 5 μCi/ml [^{14}C]glucose, or 0.8 mCi/ml $^{3}H_2O$. In the study using [^{14}C]glucose, D-glucose was not included in the culture medium. Cells were treated with compound or vehicle (0.3% DMSO) for up to 4 h followed by lipid isolation.

In vivo measurement of lipid synthesis with [^{14}C]acetate

Rats were fasted for 48 h and then refed for 48 h, with the last 2 h of refeeding involving exposure to ESP 55016 and subsequent metabolic labeling. Specifically, 25 male Sprague-Dawley [Crl: CD (SD) IGSBR] rats (five per group) weighing ~125–150 g were fasted for 48 h and subsequently refed for 48 h a purified diet containing 58% sucrose and 14% corn starch as the carbohydrate and 19% casein as the protein sources (Diet D01121101B; Research Diets, Inc., New Brunswick, NJ). Near the end of refeeding, rats in each group received a single dose of 3, 10, 30, or 100 mg/kg ESP 55016 or the CMC-Tween vehicle by oral gavage. One hour after dosing, each rat received an intraperitoneal injection of 0.3 ml of saline containing 40 μCi of [1-^{14}C]acetate. One hour later, animals were killed and blood (for serum) and liver samples were obtained and used to quantify radiolabeled saponified and nonsaponified lipid levels. This scheme resulted in a total 2 h exposure to ESP 55016.

In vitro measurement of sterol synthesis with [^{14}C]mevalonate

Lipid synthesis experiments were performed in hepatocytes cultured in DMEM with glucose and further supplemented with 100 μM *R/S*-mevalonolactone plus the appropriate metabolic precursor as follows: [^{14}C]acetate (1.0 μCi/ml, specific activity of 0.2 Ci/mol) and [^{14}C]mevalonate (1.0 μCi/ml, specific activity of 8.4 Ci/mol). Cells were treated with vehicle (1.0% DMSO), ESP 55016, or 200 nM zaragosic acid for 1 h.

In vivo measurement of sterol synthesis with [^{14}C]mevalonate

Twenty male Sprague-Dawley [Crl: CD (SD) IGSBR] rats weighing ~125–150 g were divided into four groups of five and acclimated for a minimum of 7 days to an alternating cycle of 12 h of light (4 PM to 4 AM) and darkness (4 AM to 4 PM) while having free access to water and standard Purina Laboratory Rodent Chow® 5001. After the acclimation phase, animals were given a Purina 5001 chow diet supplemented with 2.5% cholestyramine for an additional 5 days while maintaining the same light/dark cycle. Cholestyramine feeding prevents reabsorption of intestinal bile acids, thereby inducing hepatic HMG-CoA reductase activity severalfold (25). The light cycle used transfers of the natural peak of the diurnal cholesterol biogenesis cycle to ~10 AM (26). By the end of the acclimation and cholestyramine feeding phases, rats weighed an average of 280g. Animals were administered vehicle (CMC-Tween) or vehicle plus 30 mg/kg ESP 55016 by oral gavage. One hour later, animals received a single intraperitoneal injection of either sodium [1-^{14}C]acetate (40 μCi/rat) or *R/S*-[2-^{14}C]mevalonolactone (4 μCi/rat). After 1 h of isotope exposure, animals were euthanized with CO_2. Blood was collected by cardiac puncture and processed for separation of serum, and a portion of the liver was removed and frozen in liquid nitrogen to determine isotope incorporation into nonsaponified (sterols) lipids.

Isolation of saponifiable (fatty acids) and nonsaponifiable (sterols) lipids

After metabolic labeling in vitro and in vivo, saponified and nonsaponified lipids were extracted from serum, liver, and cell suspensions essentially as described by Slayback, Cheung, and Geyer (27).

Methods for acetyl-CoA carboxylase and AMP-activated protein kinase phosphorylation states

Crude hepatocyte lysates for acetyl-CoA carboxylase (ACC) and AMP-activated protein kinase (AMPK) phosphorylation state assessments were isolated with a digitonin-based lysis buffer containing 20 mM Tris (pH 7.5), 50 mM NaCl, 0.25 M sucrose, 50 mM NaF, 30 mM Na pyrophosphate, 2 mM DTT, 10 μM $ZnCl_2$, 0.1 mM Na vanadate, 0.4 mg/ml digitonin, and 1× Phosphatase Inhibitor Cocktail I (Sigma). A 35% ammonium persulfate cut was obtained from the crude lysate and resuspended in digitonin-free lysis buffer. Protein concentrations within each sample were subsequently determined using the BCA protein assay (Pierce, Rockford, IL). Thirty to 90 μg of protein from primary rat hepatocytes treated with DMSO or 10–300 μM ESP 55016 was separated by size via 4% stacking/7.5% resolving SDS-PAGE and transferred to a polyvinylidene difluoride membrane. For ACC phosphorylation, the membrane was probed with a rabbit polyclonal IgG directed against phosphorylated ACC at serine 79 (Upstate Biotech, Lake Placid, NY). For AMPK phosphorylation, the membrane was probed with a rabbit polyclonal IgG directed against phosphorylated AMPK at threonine 172 (Cell Signaling Technologies, Beverly, MA). For both proteins, bound primary antibody was captured and detected with a horseradish peroxidase-conjugated rabbit polyclonal IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and ECL + Western Blot Development Substrate (Amersham Pharmacia Biotech, Piscataway, NJ) followed by exposure to Biomax ML film (VWR Scientific Products, Chicago, IL).

Synthesis, detection, and identification of xenobiotic-CoA derivative

Rat liver microsomes were used to generate a 55016-CoA conjugate by the method of Pande and Mead (28). The reaction products were separated using a Waters Alliance 9960 HPLC device fitted with an Intersil C:18, 5 μm, 250 × 4.60 mm column (MetaChem, Lake Forest, CA). Peaks were detected at 254 nm using a Waters 2996 photodioarray detector. Elution peaks in which appearance was both time and dose dependent in the microsomal assay were collected as ESP 55016-CoA candidates and subjected to mass spectrometry. HPLC fractions were taken up in standard electrospray ionization (ESI)-positive ion buffer [water-methanol-acetic acid (48:48:4)] to a concentration of ~0.01 mg/ml acyl-CoA, as determined by the optical density at 254 nm, and infused at 60 μl/h into the Apollo ESI source of a Bruker Apex III Fourier transformed mass spectrometry instrument operating in the positive ion detection mode. Ions were accumulated for 1.5 s in the hexapole segment before injection into the cell. Transients (8–128) were accumulated using the standard SIMPLE.EXP pulse program. The resulting free induction decays (512,000 points) were apodized using a squared sine bell function, Fourier transformed, and the absolute magnitude signal was displayed. The ESP 55016-CoA species was easily detected and identity confirmed by molecular mass.

Isolation of partially purified ACC and assessment of enzyme activity

ACC was partially purified from rat liver using strepavidin-agarose, and the ACC reaction was performed essentially as described (29, 30). The reaction involved preincubation of the par-

tially purified enzyme at 37°C for 30 min in the presence of 50 mM Tris (pH 7.5), 10 mM potassium citrate, 10 mM $MgCl_2$, 1 mM DTT, and 0.075 mg/ml BSA (fatty acid free) to activate ACC followed by the addition of 4 mM ATP, 0.5 mM acetyl-CoA, and 12.5 mM [^{14}C]sodium bicarbonate (7.2 mCi/mmol) (Sigma). Incorporation of ^{14}C into acid-stable products ([^{14}C]malonyl-CoA) was determined by liquid scintillation spectroscopy. Data were transformed into micromoles of malonyl-CoA formed as a function of time to determine the reaction rate (1 unit of activity is defined as 1 μmol of malonyl-CoA formed per minute). Characterization of partially purified ACC under these reaction conditions indicated that generation of malonyl-CoA was linear for up to 10 min with 0.5 ng/μl affinity-purified protein. Further characterization indicated that the K_m values for key variables were as follows: acetyl-CoA, 26 μM; ATP, 0.2 mM; and citrate, 4 mM. The V_{max} under saturating conditions was ~4 U/mg.

RESULTS

Effect of ESP 55016 in obese female Zucker rats

The obese female Zucker rat has a mutation in the leptin receptor that leads to a human-like non-insulin-dependent diabetes mellitus with associated dyslipidemia. We assessed the effects of oral daily administration of ESP 55016 on serum lipid changes after 1 and 2 weeks at doses up to 100 mg/kg (**Fig. 2**). After 1 week of dosing, non-HDL-C was reduced at almost all doses tested (except 10 mg/kg). After 2 weeks, non-HDL-C increases of 1.38- and 1.33-fold were observed at 10 and 30 mg/kg, respectively, likely reflecting an increase in cholesterol biosynthetic enzymes and mRNAs, which have been observed with cholesterol synthesis inhibitors in rodents (31). However, at 100 mg/kg there were 81% and 80% decreases after 1 and 2 weeks of dosing, likely indicating a sufficient concentration of inhibitor to suppress the increased cholesterol biosynthetic enzyme mass. A similar but less pronounced compensation (compare 1 week vs. 2 week data) was reflected in the triglyceride response; however, a typical dose response was observed, with decreases of 30, 52, 78, and 91% at 3, 10, 30, and 100 mg/kg, after 2 weeks of treatment. HDL-C levels increased in a time- and dose-dependent manner, with increases of 1.57-, 2.45-, and 2.79-fold at 10, 30, and 100 mg/kg, respectively, after 2 weeks. ESP 55016 caused similar changes in non-HDL-C, triglycerides, and HDL-C in other animal models, including the chow-fed rat, cholesterol-fed rat, and LDL-receptor knockout mouse (data not shown). Therefore, ESP 55016 favorably alters lipids in models with either normal or deficient leptin signaling.

Nonesterified fatty acids (NEFAs) were reduced after ESP 55016 treatment and generally mirrored by a concomitant increase in serum β-hydroxybutyrate (β-HBA) levels (**Fig. 3**). At 2 weeks, NEFA levels were reduced by 30, 28, 39, 44, and 47% at 1, 3, 10, 30, and 100 mg/kg ESP 55016, respectively. Vehicle-treated control animals displayed increased serum β-HBA over the 2 week time course of the study, consistent with the progression of diabetes. At doses of 10 mg/kg or higher, there was an in-

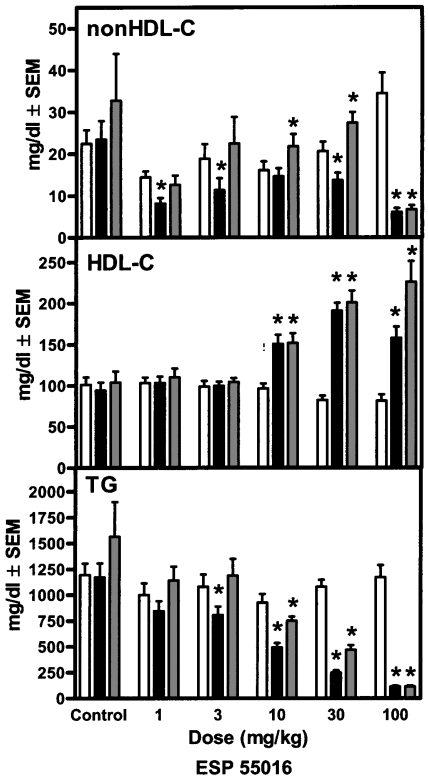

Fig. 2. Serum lipid variables in obese female Zucker rats. The data summary was compiled from four separate experiments with four to five animals per treatment group. Data are given as means ± SEM of pretreatment (white bars), at 1 week (black bars), and at the end of the 2 week dosing period (gray bars) for daily doses of 0 (control; n = 17 animals), 1 (n = 8), 3 (n = 14), 10 (n = 14), 30 (n = 16), and 100 (n = 8) mg/kg. Comparisons with pretreatment values were made using a paired Student's *t*-test. * $P < 0.05$. HDL-C, HDL-cholesterol; TG, triglyceride.

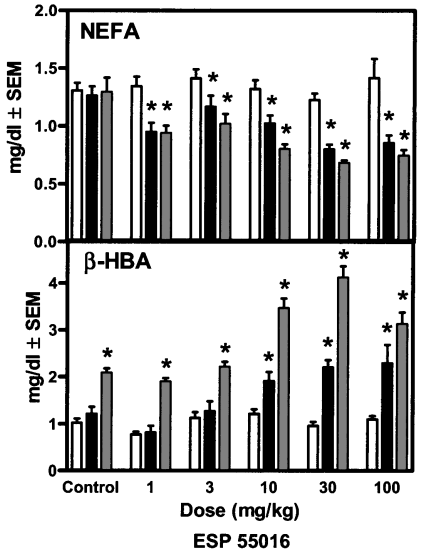

Fig. 3. Serum nonesterified fatty acid (NEFA) and β-hydroxybutyric acid (β-HBA) in obese female Zucker rats. The same samples described in the legend to Fig. 2 were used for these analyses. Comparisons with pretreatment values were made using a paired Student's *t*-test. * $P < 0.05$.

TABLE 1. Effect of ESP 55016 on fasting glucose, fasting insulin, and weight gain in the obese female Zucker rat

	Vehicle Treated			ESP 55016 Treated (100 mg/kg)		
	Pretreatment	1 Week	2 Weeks	Pretreatment	1 Week	2 Weeks
Glucose (mg/dl)	117 ± 5	120 ± 2	113 ± 3	109 ± 3	91 ± 4[a] (−17%)	94 ± 3[a] (−14%)
Insulin (ng/ml)	9.2 ± 0.6	11.0 ± 1.3	8.8 ± 1.0	11.7 ± 1.8	5.0 ± 1.0[a] (−57%)	6.2 ± 0.8[a] (−47%)
Weight (g)	403 ± 6	420 ± 7[a] (+4%)	438 ± 8[a] (+9%)	407 ± 10	406 ± 11	421 ± 11[a] (+3%)

Glucose and insulin levels in addition to body weight were determined from the same animals (100 mg/kg dose) used in the experiments depicted in Figs. 2 and 3. Numbers in parentheses indicate percentage increase or decrease compared with the pretreatment value for either the vehicle-treated or ESP 55016-treated group of animals.

[a] $P < 0.05$.

crease in serum β-HBA in the ESP 55016-treated animals above the increases observed in vehicle-treated controls. After 2 weeks of dosing at 10, 30, and 100 mg/kg, ESP 55016 increased serum β-HBA levels by 3- to 4-fold over pretreatment values.

At an ESP 55016 dose of 100 mg/kg, serum levels of glucose and insulin were markedly reduced in the fasted animal (**Table 1**). Glucose levels were reduced by 17% and 14% after 1 and 2 weeks of treatment, respectively, whereas insulin levels were reduced by 57% and 47% during the same time period; no significant changes were observed in vehicle-treated controls. ESP 55016-treated animals also gained less weight over the 2 week study period. Compared with pretreatment weight, vehicle-treated animals gained 9% of body weight and ESP 55016-treated animals gained only 3% of body weight. Long-term studies in Zucker rats demonstrated that ESP 55016 treatment dramatically reduced weight gain over a 12 week period despite no differences in food intake compared with untreated animals (data not shown). Note that it typically takes 2 days of fasting for Zucker rats to reduce serum insulin by 50% (32). These data indicate that ESP 55016 favorably alters serum lipid variables by reducing non-HDL-C, triglycerides, and NEFA while increasing HDL-C. Furthermore, ESP 55016 appears to also possess the ability to improve insulin sensitivity while suppressing body weight gain.

Effect of ESP 55016 on fatty acid oxidation in primary rat hepatocytes

Because ESP 55016 treatment of Zucker rats increased serum β-HBA, we hypothesized that the compound stimulated hepatic fatty acid oxidation. ESP 55016 dose dependently (3–30 μM) stimulated [^{14}C]palmitic acid oxidation in primary rat hepatocytes after 4 h, reaching a level 71% greater than control at 30 μM (**Fig. 4A**). ESP 55016-stimulated oxidation of [^{14}C]palmitic acid was enhanced further by L-carnitine, a substrate for carnitine palmitoyl transferase-I (CPT-I), an essential enzyme in the transport of long-chain fatty acids into the mitochondria (Fig. 4A). ESP 55016-stimulated oxidation of [^{14}C]palmitic acid was completely blocked by *R*-aminocarnitine, a CPT-I inhibitor (Fig. 4A). These data suggest that ESP 55016 increases mitochondrial β-oxidation of long-chain fatty acids through a CPT-I-dependent pathway.

To test if ESP 55016 alters β-oxidation pathways within the mitochondria, we measured the oxidation of medium-chain fatty acids in primary hepatocytes. Lauric acid (C12:0) and caprylic acid (C8:0) do not require CPT-mediated translocation into the mitochondria and thus allow the assessment of intramitochondrial β-oxidation capacity. ESP 55016 did not alter the oxidation of [^{14}C]lauric acid or [^{14}C]caprylic acid under any conditions, including the presence or absence of L-carnitine or *R*-aminocarnitine (Fig. 4B). These data suggest that ESP 55016 does not alter intramitochondrial β-oxidation pathways but rather

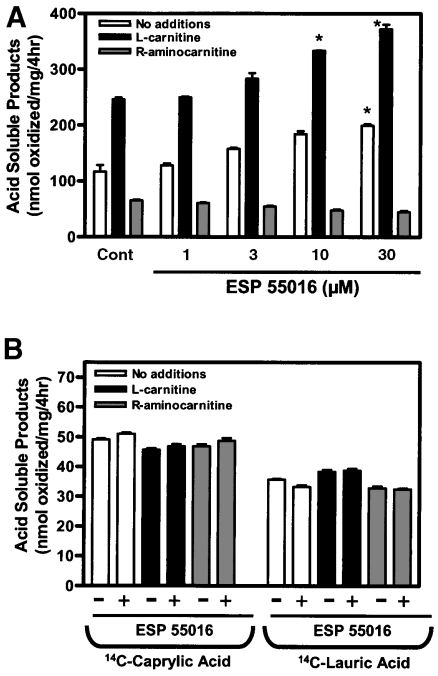

Fig. 4. Time and dose response of [^{14}C]palmitic acid oxidation to ESP 55016 treatment of primary rat hepatocytes. A: Experiments with increasing concentrations of ESP 55016 after 4 h were performed in DMSO vehicle (white bars), with 500 μM L-carnitine (black bars), and with 500 μM *R*-aminocarnitine (gray bars). Cont., control. B: Effect of ESP 55016 on the oxidation of medium-chain fatty acids. The oxidation rates of [^{14}C]caprylic acid and [^{14}C]lauric acid were assessed using DMSO control incubations (white bars), 500 μM L-carnitine (black bars), or 500 μM *R*-aminocarnitine (gray bars) in the presence or absence of 10 μM ESP 55016 as indicated. Each data point is the mean ± SEM of three wells. * $P < 0.05$.

expedites the CPT-I-dependent import of long-chain fatty acids into the mitochondria.

Effect of ESP 55016 on lipid synthesis in primary rat hepatocytes

β-Oxidation and fatty acid synthesis are opposing metabolic processes. We used four different metabolic precursors to determine if ESP 55016 inhibited fatty acid synthesis in primary rat hepatocytes. As shown in **Fig. 5**, ESP 55016 inhibited fatty acid synthesis in a dose-dependent manner when using ^3H_2O, [^{14}C]glucose, [^{14}C]pyruvate, and [^{14}C]acetate as tracers. The IC_{50} values were similar, ranging from 2 to 13 μM. Furthermore, ESP 55016 also inhibited sterol synthesis from all four precursors, with IC_{50} values ranging from 2 to 10 μM (Fig. 5). Inhibition of lipid synthesis occurred rapidly, with similar levels of inhibition observed when cells were exposed to ESP 55016 for times ranging from 5 min to 4 h (data not shown). ESP 55016 at 30 μM also inhibited triglyceride secretion by >80% in treated hepatocytes (data not shown). The rapid inhibition of fatty acid and sterol biosynthesis (minutes) suggested that ESP 55016 or its metabolites altered key enzyme activities of these pathways through a nontranscriptional mechanism.

Effect of ESP 55016 on lipid synthesis in vivo with [^{14}C]acetate

To confirm the physiological relevance of the in vitro findings, we tested whether ESP 55016 inhibited hepatic fatty acid and sterol synthesis in vivo in the fasted-refed rat. **Figure 6** indicates that ESP 55016 dose-dependently reduced the incorporation of [^{14}C]acetate into both fatty acids and sterols in serum and liver. The IC_{50} appeared to be between 3–10 mg/kg for both the liver and the serum.

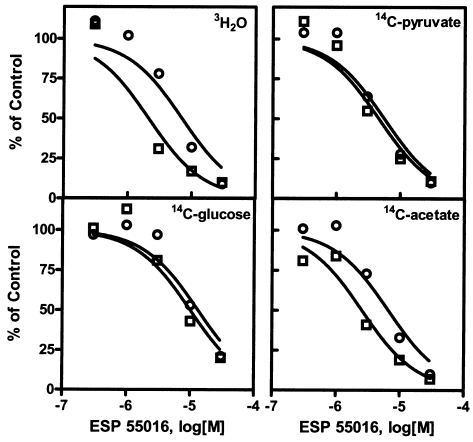

Fig. 5. Effect of ESP 55016 on fatty acid and sterol synthesis in primary rat hepatocytes. Hepatocytes from fasted rats ([^{14}C]pyruvate, [^{14}C]acetate, and ^3H_2O) or hepatocytes from fasted-refed rats ([^{14}C]glucose) were exposed to DMEM with 0.1% DMSO or 0.3, 1, 3, 10, and 30 μM ESP 55016 for 4 h in the presence of the various metabolic tracers as noted. Incorporation of radiolabeled tracer into cellular fatty acids (circles) and sterols (squares) was determined. Each data point is the mean of three wells.

Effect of ESP 55016 on [^{14}C]mevalonolactone incorporation into sterols in vitro

To further define the step(s) in the cholesterol biosynthetic pathway that may be inhibited by ESP 55016, we measured sterol synthesis in primary rat hepatocytes with the radiolabeled metabolic tracer [^{14}C]mevalonolactone. [^{14}C]mevalonolactone is readily taken up by cells and converted to [^{14}C]mevalonate for cholesterol biosynthesis. Mevalonate is the product of the reaction catalyzed by HMG-CoA reductase. Therefore, if ESP 55016 does not inhibit the incorporation of [^{14}C]mevalonate into sterols, we would deduce that the compound blocks a pathway step before mevalonate formation.

ESP 55016 had only a minor inhibitory effect (<20% inhibition at 30 μM) on the incorporation of [^{14}C]mevalonolactone into sterol lipids (**Fig. 7**); however, as previously demonstrated (Fig. 5) and as shown here (Fig. 7), the compound inhibited the incorporation of [^{14}C]acetate into sterols, with an IC_{50} of <10 μM. Zaragosic acid, a squalene synthetase inhibitor, as expected, reduced the incorporation of [^{14}C]mevalonate into sterol lipids by 80% (Fig. 7). These data suggest that in vitro, ESP 55016 inhibits sterol synthesis at one or more steps before mevalonate synthesis.

Effect of ESP 55016 on [^{14}C]mevalonolactone incorporation into sterols in vivo

To confirm the physiological relevance of our in vitro findings, we used the cholestyramine-primed rat model to induce hepatic cholesterol synthesis (33). ESP 55016 reduced the amount of radiolabeled sterols in serum by 86% compared with vehicle-treated controls when [^{14}C]acetate was used as the metabolic tracer (**Fig. 8**), confirming our earlier findings (Fig. 6). However, when [^{14}C]mevalonate was used as the metabolic tracer, there was no difference in the amount of radiolabeled sterol in the serum or liver between ESP 55016-treated and vehicle-treated animals (Fig. 8).

Effect of ESP 55016 on AMPK and ACC

Previous work by Henin et al. (34) demonstrated that activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMP analog, rapidly inhibited both fatty acid and cholesterol synthesis in primary rat hepatocytes by reducing ACC and HMG-CoA reductase activity. AMPK-catalyzed phosphorylation of key amino acid residues in either ACC or HMG-CoA reductase results in reduced enzyme activity (35, 36). Regarding ACC specifically, phosphorylation of threonine 172 of AMPK results in enhanced activity, which then leads to AMPK-catalyzed phosphorylation of ACC at serine 79. ESP 55016 did not affect the phosphorylation states of either ACC or AMPK in treated hepatocytes; AICAR, as expected (37), enhanced the phosphorylation of both enzymes (**Fig. 9**). ACC activity was also unchanged in these same extracts after ESP 55016 treatment (data not shown). In addition, extensive in vivo studies with a prodrug of ESP 55016, over a wide range of doses and times, also did not demonstrate activation of the AMPK pathway, as assessed

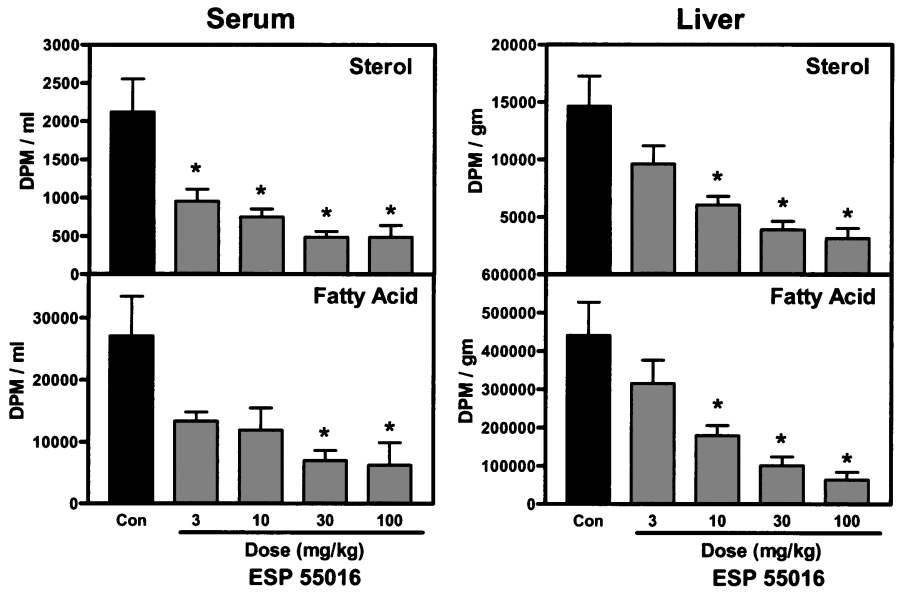

Fig. 6. In vivo inhibition of lipid synthesis by ESP 55016. Rats were fasted for 48 h followed by refeeding a high-carbohydrate diet for 48 h. On the last day of refeeding, animals received one dose of vehicle or ESP 55016 (100 mg/kg). After an additional hour, rats were injected intraperitoneally with [^{14}C]acetate, and 1 h later serum and liver were obtained to determine the incorporation into fatty acids and sterols. Five animals were used per group. Each data point is the mean ± SEM. * $P < 0.05$. Con, control.

by phosphorylation states or activities of AMPK or ACC (data not shown). Furthermore, there were no changes in AMP, ADP, or ATP levels when hepatocytes were treated with ESP 55016 (data not shown). These data strongly suggest that ESP 55016 does not inhibit lipid synthesis by activating the AMPK pathway.

Conversion of ESP 55016 to its CoA derivative

Because phosphorylation of ACC did not appear to be responsible for reduced fatty acid synthesis in vitro, we tested whether ESP 55016 or intracellular metabolites of ESP 55016 might inhibit ACC directly. Some naturally occurring fatty acid-CoA molecules inhibit ACC (38). Therefore, our initial aim was to determine whether ESP 55016 could be converted to a xenobiotic-CoA derivative using rat microsomes. Separation of reaction products by HPLC resulted in a peak with a retention time of ~8 min, the appearance of which was both incubation time and ESP 55016 (parent) concentration dependent (**Fig. 10A**). Ultraviolet spectrophotometric analysis of the reaction product at 8 min revealed a characteristic absorption spectra for a CoA-containing molecule (Fig. 10A, inset). Analysis of that peak by mass spectrometry definitively identified this chemical species as ESP 55016-CoA (Fig. 10B). To confirm that ESP 55016-CoA is formed in vivo, hepatic lipids were isolated 1.5 h after dosing rats with 100 mg/kg ESP 55016. ESP 55016-CoA was again clearly detected in this preparation, as confirmed by mass spectrometry (data not shown). The estimated ESP 55016-CoA concentration in the liver under these dosing conditions was ~2.5 nmol/mg protein. That liver concentration when converted to aqueous molarity, as described by Bronfman et al. (39), is approximately equivalent to 0.5 mM.

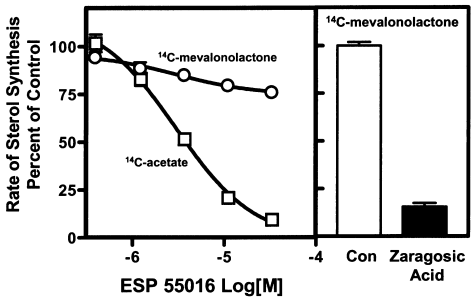

Fig. 7. Effect of ESP 55016 on the incorporation of [^{14}C]mevalonolactone into sterols in primary rat hepatocytes. Left, hepatocytes from fasted rats were exposed to DMEM with 0.1% DMSO or 0.3, 1, 3, 10, or 30 μM ESP 55016 for 4 h in the presence of [^{14}C]mevalonolactone (circles) or [^{14}C]acetate (squares). Incorporation of radiolabeled tracer into cellular sterols was determined. Right, zaragosic acid (200 nM), a squalene synthetase inhibitor, was used as a positive control for the inhibition of sterol synthesis from [^{14}C]mevalonolactone. Each data point is the mean ± SEM of three wells.

Effect of ESP 55016-CoA on ACC and HMG-CoA reductase activity in a cell-free assay

To test the inhibitory effects of ESP 55016-CoA on ACC directly, partially purified ACC was prepared. ESP 55016-CoA markedly inhibited ACC activity in a concentration-dependent manner (**Fig. 11**). Data from several experiments indicated that the IC_{50} for ESP 55016-CoA was 29 ± 5 μM (mean ± SEM, n = 7) under assay conditions that were saturating for citrate, acetyl-CoA, bicarbonate, and ATP. Similar results were obtained when either chemically

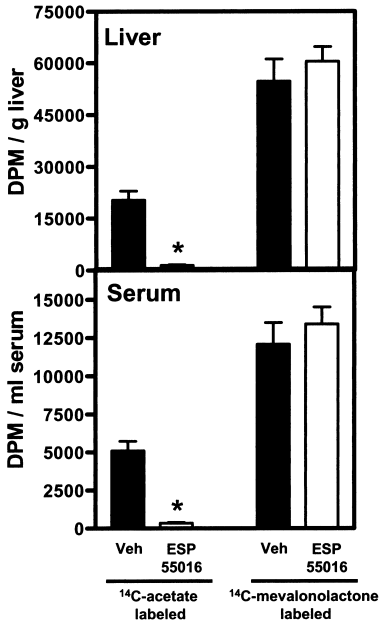

Fig. 8. Effect of ESP 55016 on the incorporation of [^{14}C]mevalonolactone into sterols in vivo. Rats were fed a cholestyramine diet for 5 days to induce hepatic cholesterol synthesis. On the last day of feeding, animals received one dose of vehicle or ESP 55016 (100 mg/kg). After an additional hour, rats were injected intraperitoneally with [^{14}C]acetate or [^{14}C]mevalonolactone, and 1 h later serum and liver were obtained to determine incorporation into sterols. Five animals were used per group. Each data point is the mean ± SEM. * $P < 0.05$. Veh, vehicle.

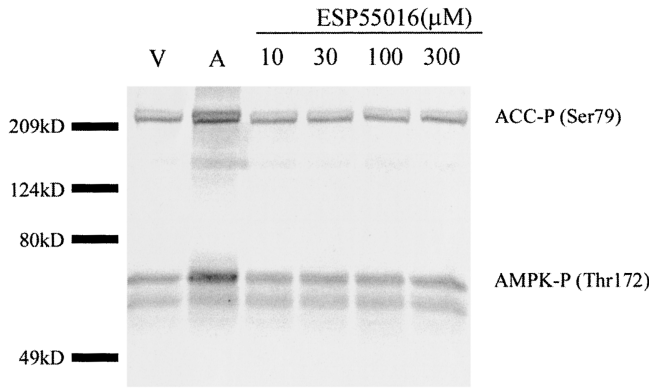

Fig. 9. Effect of ESP 55016 on the AMP-activated protein kinase (AMPK) pathway in primary rat hepatocytes. Hepatocytes were treated with DMSO [vehicle (V)], 500 μM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (A), and various concentrations of ESP 55016 for 1 h followed by the preparation of cell lysates. The phosphorylation state of acetyl-CoA carboxylase (ACC) and AMPK was determined. Cell lysates were submitted to Western blot analysis using specific antibodies to phosphorylated serine 79 of rat ACC and phosphorylated threonine 172 of rat AMPK α-subunit.

or microsomally synthesized ESP 55016-CoA was tested in this system (data not shown). The IC_{50} estimate (29 μM) was within 2-fold of the upper range for the inhibition of fatty acid synthesis in hepatocyte cultures (13 μM), with significant overlap in the 95% confidence intervals of those measurements. The parent non-CoA derivative, ESP 55016, had essentially no effect on ACC activity (Fig. 11). Palmitoyl-CoA, a known inhibitor, displayed an IC_{50} of 2 μM under these assay conditions (data not shown).

To test the effect of ESP 55016 and its CoA derivative on HMG-CoA reductase, microsomal and soluble forms of the enzyme from rat liver were prepared (40). Under conditions in which palmitoyl-CoA inhibited HMG-CoA reductase, ESP 55016 or ESP 55016-CoA did not inhibit enzyme activity (data not shown). In addition, hepatic microsomes from ESP 55016-treated hepatocytes and dosed rats displayed the same HMG-CoA reductase activity compared with untreated controls (data not shown).

DISCUSSION

We present data describing the pharmacologic activity of a novel ω-hydroxy-alkanedicarboxylic acid compound, ESP 55016, in obese female Zucker rats. ESP 55016 reduced non-HDL-C and triglycerides while increasing HDL-C. Of particular note, ESP 55016 markedly reduced serum NEFA, which was associated with an increase in the serum level of the ketone body, β-HBA.

The ESP 55016 mechanism-of-action studies were initially guided by the finding that β-HBA levels were increased. Because ketone bodies are synthesized as a result of excessive acetyl-CoA in the liver, we hypothesized that ESP 55016 enhanced the hepatic oxidation of fatty acids. Indeed, ESP 55016 enhanced the oxidation of [^{14}C]palmitate in primary rat hepatocytes. Because mitochondrial oxidation of long-chain fatty acids is mediated through CPT-I, we tested if L-carnitine, a CPT-I reaction substrate, or *R*-aminocarnitine, a CPT-I inhibitor, could modulate the ESP 55016-induced oxidation effects. ESP 55016 in the presence of L-carnitine enhanced palmitate oxidation beyond the rates observed with ESP 55016 alone. In contrast, *R*-aminocarnitine prevented the ESP 55016-enhanced oxidation of palmitate. The palmitate oxidation in the presence of *R*-aminocarnitine is presumably attributable to peroxisomal oxidation; ESP 55016 under these conditions did not appear to affect the peroxisomal oxidation pathway for palmitate. Because peroxisome oxidation pathways show a preference for fatty acids greater than chain lengths of 16 carbons (41), it is possible that peroxisomal oxidation was also enhanced. Furthermore, an increase in peroxisome-catalyzed chain shortening of palmitate could have occurred followed by mitochondrial oxidation of the resultant acyl-CoA. However, we did not test fatty acids greater than C16 in this study, nor did we monitor palmitate chain shortening outside the mitochondria. Nonetheless, these data indicate that active CPT-I is essential for the ESP 55016 stimulation of fatty acid oxidation.

McGarry and coworkers have demonstrated the intimate linkage between rates of mitochondrial fatty acid oxidation and fatty acid biosynthesis (42, 43). Malonyl-CoA is the key intracellular metabolite in that linkage and is pro-

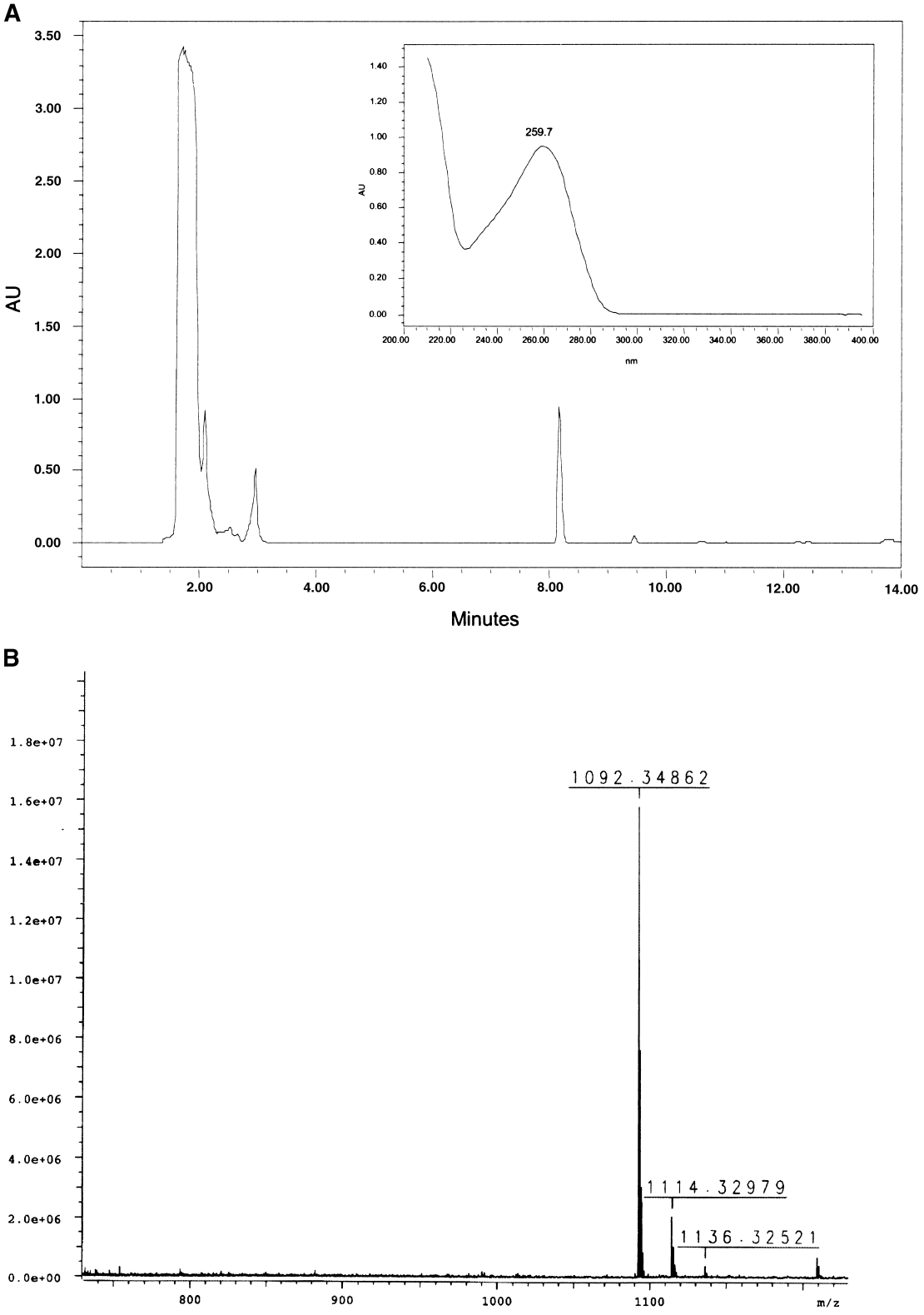

Fig. 10. Biological synthesis and identification of xenobiotic-CoA of ESP 55016. A: HPLC analysis of the product from microsomal incubation with ESP 55016. The ESP 55016-CoA peak elutes with a retention time of 8.1 min. The inset shows the ultraviolet absorption spectrum of that peak. B: A representative negative ion mode mass spectrum showing a *m/z* [M-H]$^-$ of 1,092.34862 of the purified 8.1 min peak, corresponding to the predicted *m/z* for ESP 55016-CoA. Additional monosodiated and disodiated forms of ESP 55016-CoA are present, corresponding to *m/z* [M-H]$^-$ of 1,114.32979 and 1,136.32521, respectively. AU, absorbance units.

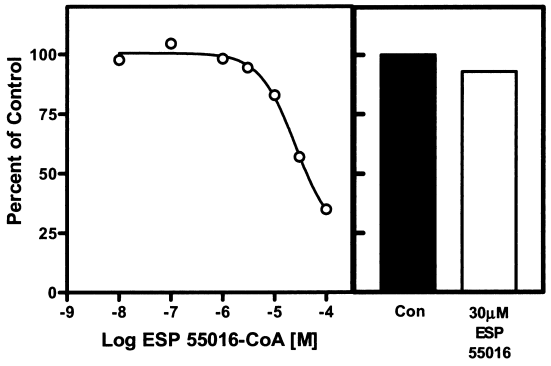

Fig. 11. Xenobiotic-CoA inhibition of partially purified ACC. ESP 55016-CoA was synthesized in a microsomal system, purified, and incubated with partially purified rat liver ACC in a cell-free assay. Parent ESP 55016 was tested at a concentration of 30 μM. These results are from a representative experiment, with several experiments under these assay conditions indicating an IC_{50} of 29 ± 5 (mean ± SEM, n = 7). Con, control.

duced through the carboxylation of acetyl-CoA catalyzed by ACC, which is widely regarded as the rate-limiting step of long-chain fatty acid biosynthesis (44). Malonyl-CoA is a potent inhibitor of CPT-I; thus, inhibition of fatty acid synthesis from acetyl-CoA reduces malonyl-CoA levels and stimulates fatty acid oxidation through derepression of CPT-I. We hypothesized that ESP 55016 inhibited fatty acid synthesis by inhibiting ACC. In primary hepatocytes, ESP 55016 inhibited fatty acid synthesis at concentrations that stimulated palmitate oxidation. Although these data suggest that ESP 55016 stimulates fatty acid oxidation by reducing malonyl-CoA levels, it does not rule out the possibility that the xenobiotic-CoA of ESP 55016 can overcome the malonyl-CoA suppression of CPT-I; fatty acyl-CoAs have been shown to relieve the inhibition of CPT-I by malonyl-CoA (45, 46).

In the course of the fatty acid biosynthesis studies, we observed that ESP 55016 also inhibited sterol synthesis from various metabolic precursors, suggesting that ESP 55016 affected a common or multiple biochemical control point(s) shared by both the fatty acid and sterol biosynthetic pathways. One such control point could have involved AMPK.

AMPK can phosphorylate ACC and HMG-CoA reductase at key amino acid phosphorylation sites to reduce enzyme-specific activity and inhibit the synthesis of fatty acids and sterols, respectively (34–36, 47). Recently, particular emphasis has focused on the AMPK and ACC tandem as a result of their demonstrated roles in disorders of the metabolic syndrome, including obesity, insulin resistance, and dyslipidemia (48, 49). The AMPK pathway can be activated by hormones such as leptin and adiponectin and by the pharmacologic agents metformin and AICAR (37, 50–52). These diverse agents have been shown to activate AMPK, stimulate the phosphorylation of ACC, reduce fatty acid synthesis, and enhance fatty acid oxidation. When we tested the ability of ESP 55016 to activate this pathway in primary hepatocytes, no activation was observed.

To further define the mechanism of action for ESP 55016, we focused on ACC and its regulation. ACC is regulated by long-term control mechanisms involving changes in enzyme levels and gene expression as well as short-term control mechanisms including covalent modification (phosphorylation/dephosphorylation) and allosteric effectors (53, 54). Time-course studies with ESP 55016 indicated that short-term mechanisms were primarily responsible for the inhibition effects. Because covalent modification of ACC phosphorylation sites (35) did not appear to be affected, we tested and confirmed that ESP 55016-CoA can inhibit ACC directly. That the xenobiotic-CoA is relevant to the mechanism of action was further confirmed by the identification of near millimolar levels of xenobiotic-CoA in liver samples at 1.5 h after a 100 mg/kg dose, which is within the time frame in which we observed the inhibition of fatty acid synthesis in vivo; this concentration is well within the range we identified for the inhibition of ACC (29 μM). These data suggest that ESP 55016 ultimately inhibits fatty acid synthesis by following a pathway that includes its conversion to a xenobiotic-CoA and direct inhibition of ACC by that xenobiotic-CoA. The generation of xenobiotic-CoA derivatives from carboxylic acid-containing compounds is well documented (55). For example, MEDICA 16 forms a CoA derivative and that metabolite inhibits ACC in a cell-free assay (20). In summary, our interpretation leads to the following scenario regarding the ESP 55016 mechanism of action with regard to fatty acid synthesis: ESP 55016 is converted to ESP 55016-CoA, which directly inhibits ACC and reduces malonyl-CoA levels, perhaps locally near CPT-I, and results in derepression of CPT-I, increased transfer of long-chain fatty acids, and enhanced fatty acid oxidation. The continuation of this state with daily dosing of ESP 55016 in animals leads to adaptations resulting in altered lipid and insulin levels as well as the suppression of weight gain.

The mechanism responsible for the inhibition of sterol synthesis is less clear than the mechanism responsible for the inhibition of fatty acid synthesis. Our studies with radiolabeled metabolic tracers indicate that inhibition of the sterol biosynthetic pathway occurs after the formation of acetyl-CoA but before the formation of mevalonate. Thus, three enzymes are potential targets for ESP 55016 or the corresponding CoA: acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase. We focused on HMG-CoA reductase for two reasons. First, reductase activity is directly inhibited by CoA thioesters (40, 56, 57); second, the hypolipidemic compound S-2E is converted to a CoA derivative and S-2E-CoA directly inhibits HMG-CoA reductase in a cell-free assay (58). However, we found that ESP 55016-CoA did not inhibit reductase in a cell-free assay under a variety of conditions. Furthermore, ESP 55016 treatment of hepatocytes and Sprague-Dawley rats followed by the isolation of microsomes and ex vivo assay of reductase activity did not reveal any differences compared with untreated controls. It is possible that the removal of HMG-CoA reductase from the cellular environment removes the pertinent regulatory mechanism. With respect to HMG-CoA synthase, it seems unlikely that activity

would be inhibited by ESP 55016 or its CoA metabolite, because ketone body levels are increased and HMG-CoA synthase is required for the synthesis of HMG-CoA, which is then used to produce acetoacetate and subsequently converted to acetone and β-HBA. However, it is conceivable that ESP 55016 or its CoA derivative inhibits acetoacetyl-CoA thiolase, but that too would limit substrate to HMG-CoA synthase, resulting in reduced ketone body synthesis. At present, we conclude that ESP 55016 or its CoA derivative inhibits the sterol biosynthetic pathway between acetyl-CoA and the production of mevalonate, possibly through an indirect mechanism.

There are alternative mechanisms that may explain or at least contribute to the overall dual inhibitor activity of ESP 55016. First, it is possible that ESP 55016 or its CoA derivative inhibits ATP-citrate lyase, an enzyme that generates cytosolic acetyl-CoA from mitochondria-derived citrate. Such a conclusion may be valid had we only measured fatty acid and sterol synthesis using [^{14}C]pyruvate, [^{14}C]glucose, or $^{3}H_2O$; all of these precursors would generate radiolabeled, mitochondria-derived acetyl-CoA. However, we used radiolabeled acetate as well and found very similar IC_{50} values for the compound with respect to its dual inhibitor property using all radiolabeled tracers. Several studies have demonstrated that the inhibition of ATP-citrate lyase with hydroxycitrate did not reduce the incorporation of [^{14}C]acetate into fatty acids or sterols, suggesting that exogenous [^{14}C]acetate does not pass through the mitochondria (59–62). Nonetheless, it is conceivable that [^{14}C]acetate could generate mitochondria-derived [^{14}C]acetyl-CoA in the liver by one or more pathways. One pathway could involve the ligation of acetate to CoA by acetyl-CoA synthetase 1 (AceCS1), transport into the mitochondria, condensation with oxaloacetate to form citrate, followed by regeneration of acetyl-CoA from citrate in the cytosol through the action of ATP-citrate lyase. An alternative mitochondria-based pathway for producing [^{14}C]acetyl-CoA from [^{14}C]acetate could involve direct ligation of [^{14}C]acetate to CoA in hepatic mitochondria. However, this seems unlikely because AceCS2, a mitochondrial matrix enzyme that is abundant in muscle, is absent in liver (63). In contrast, AceCS1 is abundant in liver and located in the cytosol, with the acetyl-CoA generated used for fatty acid and sterol synthesis (64, 65). Clearly, AceCS activity is not needed for the ESP 55016 effects, because the pathway from mitochondrial [^{14}C]pyruvate to [^{14}C]acetyl-CoA in the cytosol does not require AceCS but rather pyruvate dehydrogenase and ATP-citrate lyase (i.e., there is no free acetate generated in this process). Nonetheless, we cannot definitively rule out the possibility that ESP 55016 or ESP 55016-CoA inhibits ATP-citrate lyase to some degree. Indeed, MEDICA 16 has been shown to inhibit both ATP-citrate lyase and ACC (15, 20). In all, our data suggest that ESP 55016 inhibits both pathways at steps after the generation of acetyl-CoA.

Another possible mechanism to explain the dual inhibitor activity of ESP 55016 is that the compound increases the cellular pool size of the corresponding "cold" metabolic precursor used in the metabolic labeling studies, thus reducing the specific activity of the exogenously added radiolabeled tracer. However, as noted above, the dual inhibition occurred within minutes, making it unlikely that there were significant changes in intracellular pool sizes of experimentally related metabolites. In addition, the cell culture medium contained millimolar concentrations of acetate and pyruvate, providing a very large pool that is presumably in equilibrium with cellular levels. Finally, all of the radiolabeled tracers used showed similar inhibitory effects, including $^{3}H_2O$, which does not suffer from pool dilution effects.

It is possible that ESP 55016 sequesters CoA, limiting the availability of acetyl-CoA for lipid synthesis. This appears to be the case with TOFA at high concentrations (66). However, at concentrations at which ESP 55016 inhibited lipid synthesis, fatty acid oxidation was increased, suggesting that CoA was not limiting for the generation of palmitoyl-CoA from palmitate. Furthermore, the oxidation rates of medium-chain fatty acids, caprylic and lauric acid, were not altered by ESP 55016 treatment, again indicating that CoA was not limiting.

Finally, changes in gene expression may be responsible, in part, for long-term (days) effects of ESP 55016 in vivo. The peroxisome proliferator-activated receptor and sterol-regulatory element binding protein transcription factors regulate the expression of key enzymes involved in lipid synthesis and oxidation. Perhaps ESP 55016 metabolites or cellular lipid changes induced by ESP 55016 alter the activities of key transcription factors. Further studies are required to address these possibilities.

ESP 55016 appears to have similar short-term (minutes to hours) biochemical effects as other long-chain hydrocarbon compounds. MEDICA 16, TOFA, and 3-thiadicarboxylic acid all enhance fatty acid oxidation and inhibit fatty acid biosynthesis to various degrees (3, 4, 67). In each case, the CoA derivative is formed, and in some instances, direct effects on ACC have been demonstrated (3, 20, 68). Specifically with respect to ESP 55016, the "mechanistic anchor" contributing to in vivo alterations in lipid metabolism appears to be its ability to function as a dual inhibitor of fatty acid and sterol synthesis.

The utility of ESP 55016 to treat human dyslipidemias is unclear. De novo fatty acid synthesis (lipogenesis) in humans is considered a quantitatively minor pathway for the synthesis of liver-derived fatty acids and triglycerides under normal dietary conditions (69). Nonetheless, de novo fatty acid synthesis in humans may primarily serve a regulatory function to control the rate of fatty acid oxidation, particularly in muscle (69, 70). Whether ESP 55016 will be useful to treat human dyslipidemias and metabolic syndrome-related disorders must wait clinical testing.

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