Hypolipidemic action of the SERM acolbifene is associated with decreased liver MTP and increased SR-BI and LDL receptors

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Abstract This study aimed to identify the mechanisms of the hypolipidemic action of the selective estrogen receptor modulator (SERM) acolbifene (ACOL). Four weeks of treatment with ACOL reduced fasting and postprandial plasma triglycerides (TGs), an effect associated with lower VLDL-TG secretion rate (-25%), and decreased mRNA of microsomal triglyceride transfer protein (MTP; -29%). ACOL increased liver TG concentration ($+100\%$) and amplified the feeding-induced increase in the master lipogenic regulators sterol-regulatory element binding protein-1a (SREBP-1a) and SREBP-1c. ACOL decreased total, HDL, and non-HDL cholesterol (CHOL) by 50%. SREBP-2 mRNA and HMG-CoA reductase activity were minimally affected by ACOL. However, in the fasted state, liver concentration of scavenger receptor class B type I (SR-BI) protein, but not mRNA, was 3-fold higher in ACOL-treated than in control animals and correlated with plasma HDL-CHOL levels ($r = 0.80$, $P < 0.002$). Liver LDL receptor (LDLR) protein, but not mRNA, was increased 2-fold by ACOL, independently of the nutritional status. This study demonstrates that ACOL possesses the unique ability among SERMs to reduce VLDL-TG secretion, likely by reducing MTP expression, and strongly suggests that the robust hypocholesterolemic action of ACOL is related to increased removal of CHOL from the circulation as a consequence of enhanced liver SR-BI and LDLR abundance.—Lemieux, C., Y. Gélinas, J. Lalonde, F. Labrie, K. Cianflone, and Y. Deshaies. **Hypolipidemic action of the SERM acolbifene is associated with decreased liver MTP and increased SR-BI and LDL receptors.** *J. Lipid Res.* 2005. **46:** **1285–1294.**

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The concerns raised by the Women's Health Initiative Study regarding the health risks/benefits of hormone replacement therapy in postmenopausal women (1–3) strengthen the interest in evaluating the metabolic properties of other steroids as well as selective estrogen receptor modulators (SERMs) to improve the quality of life of this population. The estrogen antagonist acolbifene (ACOL; EM-652.HCl) is a SERM that was developed for the prevention and treatment of estrogen-sensitive cancers (4, 5). The compound behaves as a highly potent and pure antiestrogen in human breast and uterine cancer cells in vitro as well as in vivo in nude mice (4, 5). ACOL binds selectively to both the α and β types of estrogen receptors (6). Despite its pure antiestrogenic activity in the mammary gland and endometrium, ACOL can be classified as a SERM based on some estrogen-like properties, such as prevention of bone loss, decreasing plasma lipids (7–9), and reduction in body weight (mostly fat) gain (9, 10) in animal models. Therefore, ACOL shares actions on energy balance and lipemia with other steroids, such as various anticarcinogenic agents (11–15) and dehydroepiandrosterone (16–19).

One of the most striking actions of ACOL in rodent models is its robust hypolipidemic action. In rats, a triglyceride (TG)-lowering action has been reported in freely fed rats treated for 9 months with the ACOL prodrug EM-800 (8). Also, ACOL was found not to increase triglyceridemia, compared with estradiol treatment, in ovariectomized rats (10). Therefore, ACOL may beneficially affect triglyceridemia, at least under some conditions, an effect that would clearly diverge from that of other antiestrogens and of estrogen itself (2, 5, 8). Regarding cholesterolemia, we (9, 10) and others (7, 8) have shown a reduction of up to 50% in

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plasma cholesterol (CHOL), including in the HDL fraction, after chronic treatment of rats with ACOL. The mechanisms whereby ACOL affects plasma lipids are unknown. The ACOL-induced reduction in food intake (9, 10) obviously decreases overall lipid flux, but food restriction to the levels of ACOL-treated rats does not reduce plasma CHOL (C. Lemieux and Y. Deshaies, unpublished observations), suggesting peripheral mechanisms of action.

The TG-lowering effect of ACOL discussed above was not observed in fasted rats (9). This led to the hypothesis that the TG-lowering action of ACOL may depend on nutritional status. Estrogen favors hypertriglyceridemia through an increase in hepatic VLDL secretion (20). Whether ACOL affects TG appearance in or clearance from the circulation is unknown. The hypocholesterolemic effect of pharmacological doses of estrogen in rodent models has been ascribed to an increase in LDL receptor (LDLR) expression in the liver (21, 22). In contrast to the LDLR, the liver scavenger receptor class B type I (SR-BI), which plays a key role in reverse CHOL transport (23–28), is strongly downregulated by estrogen in the rat (29, 30). Because ACOL is a SERM, it appeared of primary interest to determine whether or not the drug exerts estrogen-like actions on these liver receptors as well as on key determinants of hepatic CHOL synthesis and metabolism.

Because of these considerations, the present study was designed to assess the metabolic effects of ACOL under controlled conditions of short-term fasting and acute refeeding and focused on several key determinants of the production and clearance of CHOL- and TG-rich lipoproteins.

MATERIALS AND METHODS

Animals and treatments

Sixty female Sprague-Dawley rats initially weighing 150–175 g were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) and housed individually in stainless steel cages in a room kept at $23 \pm 1°C$ with a 12 h/12 h light/dark cycle (lights on at 7:00 PM). The animals were cared for and handled in conformance with the Canadian Guide for the Care and Use of Laboratory Animals, and the experimental procedures were approved by our institutional animal care committee. The animals were acclimated to their environment for 1 week, during which they had ad libitum access to tap water and a nonpurified rodent diet. They were then fed ad libitum a purified high-carbohydrate, essentially CHOL-free diet. The diet contained (in grams per 100 g of diet) starch, 31.2; dextrose, 31.2; corn oil, 6.4; casein, 20.0; DL-methionine, 0.3; vitamin mix (Teklad 40060; Teklad Test Diets, Madison, WI), 1.0; mineral mix (AIN-76; ICN Biochemicals, Aurora, OH), 4.9; and fiber (Alphacel; ICN Biochemicals), 5.0. The diet contained 0.3 mg/g phytosterol, no CHOL, and provided 64.9% of energy as carbohydrate, 14.5% as fat, and 20.6% as protein, with an energy density of 16.8 kJ/g. Rats were divided into two groups (control and ACOL). ACOL was given once daily at 7:30 PM by oral gavage at a dose of 0.5 mg/rat (average of ~2.5 mg/kg) in a total volume of 0.5 ml of a methylcellulose suspension. This dose of ACOL prevents tumor growth in rats (4). The control group was given the vehicle alone. Treatment was administered for 28 days. Food intake and body weight were monitored every other day. On the day before the end of the treatment period, food was removed immediately after the 7:30 PM gavage. Food was returned to the cages the next morning after a 12.5 h fast, for a maximum period of 2 h, to avoid any residual effect of ACOL on later food intake. Six rats in each group were killed at 0 (fasting), 1, 2, 3, and 6 h after the onset of the 2 h refeeding period.

Blood and tissue collection

Rats were killed by decapitation, and blood was collected from the neck wound. Blood was immediately centrifuged at 1,500 g at 4°C for 15 min. Serum was stored at $-80°C$ for later biochemical measurements. Retroperitoneal and inguinal white adipose tissues, interscapular brown adipose tissue, the soleus and red vastus lateralis muscle, the heart, and the liver were excised and weighed, and samples were processed for later determination of LPL activity as described previously (31).

Serum/plasma measurements

Serum glucose concentration was measured with the Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). Serum insulin was determined by radioimmunoassay using a reagent kit from Linco Research (St. Charles, MO) with rat insulin as standard. An index of fasting insulin sensitivity, consisting of the product of fasting serum glucose and insulin, was calculated according to the homeostasis model assessment of insulin resistance (HOMA-IR) described by Matthews et al. (32). Plasma TGs were essayed by an enzymatic method using a reagent kit from Boehringer Mannheim (Montréal, Quebec, Canada) that allows correction for free glycerol. Serum nonesterified fatty acids were determined by an enzymatic colorimetric technique using a reagent kit from Wako Pure Chemical Industries (Richmond, VA). Serum total and HDL-CHOL were quantified using a reagent kit from Boehringer Mannheim. The HDL fraction was isolated by precipitation of apolipoprotein B-containing lipoproteins with sodium phosphotungstate-magnesium chloride. Non-HDL CHOL was obtained by difference.

In vivo TG kinetics

The in vivo TG clearance rate and VLDL-TG secretion rate were determined in a separate cohort of animals. Forty rats identical to the cohort described above were cared for and treated exactly as described above. Two weeks into the treatment period, rats were fitted with a permanent polyethylene cannula in the right jugular vein under isoflurane anesthesia. They were then allowed to recover for 3 days until the TG clearance rate procedure, followed by 2 days of recovery until the VLDL-TG secretion rate procedure. Half of the animals were assessed after a 12 h fast, whereas the other half were studied after 2 h of refeeding that followed a 12 h fast. Rats were refed their habitual diet from which corn oil had been removed to avoid inflow of dietary lipids and chylomicron formation, thereby allowing quantitation of VLDL-associated TG secretion and clearance of an Intralipid emulsion (Vitrum, Stockholm, Sweden). VLDL-TG secretion rate was assessed by the Triton WR-1339 method (33), whereas TG clearance rate was quantified using a bolus administration of 20% Intralipid (34). Fasting and postprandial triglyceridemia levels reported here are those measured in plasma samples taken immediately before Intralipid injection.

Tissue lipoprotein lipase activity

Lipoprotein lipase activity in inguinal and retroperitoneal white and brown adipose tissue, skeletal muscle, and heart was measured as described previously (31) by quantifying the amount of fatty acids hydrolyzed from a labeled triolein-containing substrate by tissue homogenates.

Liver lipid content

Frozen liver samples were thawed, and total lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (35)

and solubilized in isopropanol. Liver CHOL and TG were quantified in the lipid extracts using the reagent kits described above.

Western blot analysis of SR-BI, LDLR, and peroxisome proliferator-activated receptor α

Liver content of these proteins was determined in liver extracts, after electrophoretic separation, by radioimmunoassay using commercially available antibodies or, in the case of the LDLR, an antibody kindly provided by Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX). To extract the SR-BI protein, a liver sample (~50 mg) was homogenized in buffer A containing 20 mM Tris-HCl (pH 7.5), 2 mM MgCl$_2$, 0.2 M sucrose, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 5 μg/ml pepstatin A. The crude extract was centrifuged at 10,000 g for 10 min at 4°C to remove tissue debris. The supernatant was then ultracentrifuged at 100,000 g for 45 min at 4°C. The pelleted membrane fraction was then resuspended in buffer B containing 62.5 mM Tris-HCl (pH 6.8), 2% (v/v) SDS, 10% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol and frozen at −80°C until further processing. To extract the LDLR protein, a liver sample (~100 mg) was homogenized in 1 ml of buffer C containing 50 mM Tris (pH 7.5), 2 mM CaCl$_2$, 0.5% Triton X-100, and 1 mM each of leupeptin, PMSF, and phenanthroline. The crude extract was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was then ultracentrifuged at 100,000 g for 60 min at 4°C. The supernatant fraction was frozen at −80°C until further processing. To extract the peroxisome proliferator-activated receptor α (PPARα) protein, liver (~90 mg) was homogenized in 11 volumes of buffer D containing 50 mM HEPES (pH 7.4), 250 mM sucrose, 4 mM MgCl$_2$, 1 mM PMSF, 1 mM benzamide, 1 mM Na$_3$VO$_4$, and 2 mM NaF. The crude extract was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was frozen at −80°C until further processing. Protein concentration of the liver extracts was determined by the method of Lowry et al. (36). Five micrograms of SR-BI, 10 μg of LDLR, and 50 μg of PPARα protein extracts per lane were loaded on 7.5% polyacrylamide gels and separated by electrophoresis under nonreducing conditions. The protein bands were transferred onto polyvinylidene difluoride membranes, which were incubated overnight with 1:1,500 anti-SR-BI (Novus Biologicals, Inc., Littleton, CO), 1:10,000 anti-LDLR (kindly provided by Dr. Joachim Herz), or 1:150 anti-PPARα (Santa Cruz Biotechnology, Santa Cruz, CA), then with 1:10,000 horseradish peroxidase-linked anti-rabbit IgG (Amersham Biosciences UK Ltd., Little Chalfont, UK), and developed with the chemiluminescent agent ECL+ (Amersham Biosciences) and exposed to autoradiographic film (Kodak BioMax MR film).

Sterol regulatory element binding proteins 1a, 1c, and 2 mRNA

The sterol-regulatory element binding protein (SREBP) mRNA transcripts were quantified by RNase protection assays with probes specific for each SREBP. The cDNA probe for rat SREBP-1a, SREBP-1c, and SREBP-2 was generated as described by Shimomura et al. (37). The cDNA fragment for rat SREBP-1a and SREBP-1c was amplified by RT-PCR from first-strand cDNA using rat liver total RNA as a template and primers derived from the rat SREBP-1a sequence (5′ primer, 5′-ATGGACGAGCTGCCCTTCGGTGAGG-CGGCT-3′; 3′ primer, 5′-CCAGAGAGGAACCCAGGGAAGCAG-3′). The amplified fragment contains exon 1a (specific for SREBP-1a) and part of exon 2 (common to SREBP-1a and SREBP-1c). The cDNA fragment for rat SREBP-2 was amplified by RT-PCR from first-strand cDNA using rat liver total RNA as a template and primers derived from the rat SREBP-2 sequence (5′ primer, 5′-GAG-CTGACTCTCGGGGACAT-3′; 3′ primer, 5′-ACTGCCGCCACCAC-CTCCAG-3′). The 18S probe was from Ambion. From a total sample of 60 μg of RNA extracted in Trizol (Invitrogen, Burling-

ton, Ontario, Canada) from ~100 mg of liver, 10 μg (SREBP-1a and SREBP-1c), 20 μg (SREBP-2), and 1 μg (18S) of total RNA were subjected to the RNase protection assay. After digestion by A/T1, protected fragments were separated on an 8 M urea/5% polyacrylamide gel and subjected to autoradiography. Protected fragments corresponding to rat SREBP-1a (257 bp), SREBP-1c (150 bp), and SREBP-2 (120 bp) were quantified with an Imaging Densitometer (Bio-Rad). Labeling intensity of 18S (80 bp) in each sample was used to normalize signals obtained for the SREBP-1a, SREBP-1c, and SREBP-2 mRNAs. After quantitation of SREBP-1c, autoradiography of the gel was continued to achieve sufficient resolution of SREBP-1a, which was then normalized with 18S values obtained at the time of SREBP-1c quantitation.

LDLr, SR-BI, and microsomal triglyceride transfer protein mRNA

These transcripts were quantified by real-time PCR. Total RNA was prepared from liver using the Trizol RNA extraction method. RNA concentration was estimated from absorbance at 260 nm, and RNA was reverse-transcribed using Expand reverse transcriptase (Roche Diagnostics, Laval, Quebec, Canada). The expression level of mRNA was quantitated using quantitative fluorescent real-time PCR according to the manufacturer's instructions (Corbett Research, New South Wales, Australia). Amplification and detection of the target mRNA was performed with Platinum Taq polymerase and the intercalating dye Sybr-Green I. The primers, designed using the Vector NTI program and synthesized by Invitrogen, were as follows. For LDLr: 5′ primer, 5′-AAGGCTGTGGGTTC-CATAGG-3′; 3′ primer, 5′-TGGACCCTTTCTCTCGGAAC-3′. For SR-BI: 5′ primer, 5′-GTAGTAAAAAGGGCTCGCAG-3′; 3′ primer, 5′-AACAGGCTCTACTCAGCAGC-3′. And for microsomal triglyceride transfer protein (MTP): 5′ primer, 5′-GCTGGAAGGCT-TAATTGCAG-3′; 3′ primer, 5′-CGGGTTTTAGACTCGCGGTA-3′. The mRNA levels of LDLr, SR-BI, and MTP were normalized to the amount of L27 mRNA (a gene not affected by treatments) detected in each sample, and results are expressed as gene/L27 mRNA.

HMG-CoA reductase and ACAT activities

The activity of HMG-CoA reductase was determined by quantifying the production of mevalonate from labeled HMG-CoA by tissue extracts (38), and that of ACAT was determined by assessing the esterification of CHOL with labeled oleate (39).

Statistical analysis

Data are expressed as means ± SEM. For cumulative variables related to the 4 week treatment with ACOL (**Table 1**), data from all prerefeeding and postrefeeding time points in the control and ACOL-treated groups were pooled and analyzed by unpaired Student's t-test. For variables assessed at various prerefeeding and postrefeeding time points, main and interactive treatment effects were analyzed using a 2 × 5 factorial ANOVA with two factors: drug with two levels (placebo, ACOL), and time with five levels (0, 1, 2, 3, and 6 h after onset of refeeding). Fasting and postprandial TG clearance and VLDL-TG secretion rates were analyzed by a 2 × 2 factorial ANOVA with two factors: drug (placebo, ACOL), and nutritional status (fasted, fed). Differences were considered statistically significant at $P < 0.05$. The analyses were performed using StatView version 5.0.1 software.

RESULTS

The effects of a 4 week treatment with ACOL on variables related to body composition are summarized in Ta-

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TABLE 1. Final body weight and gain, cumulative food intake, and tissue weights of rats treated or not with ACOL for 4 weeks

Variable	Placebo	ACOL	P
Body weight (g)	243 ± 3	224 ± 2	<0.0001
Weight gain (g)	67 ± 3	49 ± 1	<0.0001
Cumulative food intake (g)	269 ± 5	249 ± 5	0.004
Food efficiency (%)[a]	24.7 ± 0.9	19.6 ± 0.6	<0.0001
Inguinal white adipose tissue (g)	1.25 ± 0.07	0.72 ± 0.04	<0.0001
Retroperitoneal white adipose tissue (g)	1.73 ± 0.13	0.86 ± 0.07	<0.0001
Interscapular brown adipose tissue (g)	0.50 ± 0.02	0.36 ± 0.02	<0.0001
Soleus (g)	0.112 ± 0.002	0.103 ± 0.004	0.05
Vastus lateralis (g)	0.96 ± 0.02	0.97 ± 0.04	NS
Heart (g)	0.91 ± 0.02	0.84 ± 0.02	0.0008

ACOL, acolbifene. Values are means ± SEM of 28–30 animals.
[a] Food efficiency, grams of weight gain/100 g of food ingested.

ble 1. ACOL reduced final body weight (−8%) and weight gain (−27%), effects that were associated with decreases in food intake (−7%) and food efficiency (−21%). The ACOL-induced decrease in weight gain was largely linked to fat mass, as indicated by a 50% reduction in both subcutaneous (inguinal) and visceral (retroperitoneal) adipose tissue weight, whereas muscle weights were much less affected.

Because insulin sensitivity strongly affects lipid metabolism, we took the opportunity of the present fasting-refeeding paradigm to assess glucose and insulin responses to the physiologic stimulus of meal intake. The amount of food ingested during the 2 h refeeding period was not affected by chronic ACOL administration [placebo, 3.7 ± 0.4; ACOL, 4.1 ± 0.3 g; n = 18 each (2, 3, and 6 h groups); NS]. As shown in **Table 2**, ACOL ameliorated indices of insulin sensitivity. After a 12 h fast, the HOMA-IR was 41% lower in ACOL-treated than in control rats. Upon refeeding, glycemia increased similarly in both groups, whereas insulin excursion was greatly decreased in ACOL-treated animals compared with controls.

The effects of chronic ACOL treatment on major determinants of triglyceridemia were determined in the fasted and fed states in rats refed a diet devoid of fat; these results are depicted in **Fig. 1**. In both nutritional states, triglyceri-demia was reduced by ACOL (Fig. 1A). The drug significantly reduced the secretion rate of VLDL-TG in the circulation, both in the fasted and fed states (Fig. 1B), and tended ($P = 0.06$) to increase the rate of intravascular TG hydrolysis (Fig. 1C). In rats refed their habitual fat-containing diet, mRNA levels of MTP, an essential enzyme in apolipoprotein B lipidation and VLDL assembly, were significantly reduced by ACOL treatment (Fig. 1D). The postprandial increase in retroperitoneal adipose tissue LPL activity (identical results in inguinal LPL are not shown), a major determinant of TG clearance, expressed per total fat depot to reflect global availability, was substantially blunted by chronic ACOL treatment (Fig. 1E). Such reduction was proportional to that in fat mass, because LPL per unit tissue weight or per unit total protein was similar in both groups (data not shown). In other major sites of TG clearance, including brown adipose tissue, soleus and vastus lateralis muscles, and heart, LPL activity was minimally affected by refeeding and chronic ACOL treatment (data not shown).

The responses of serum total and lipoprotein CHOL concentrations to refeeding and to chronic ACOL treatment as well as the lipid content of the liver are shown in **Fig. 2**. As expected with a CHOL-free diet, changes in CHOL concentrations elicited by acute refeeding were modest.

TABLE 2. Fasting insulin resistance index HOMA-IR, and serum glucose and insulin concentrations, after a 12 h fast (0 h time point) and 1, 2, 3, or 6 h after the onset of a 2 h refeeding period in rats treated or not with ACOL for 4 weeks

| Variable | Hours after Onset of Refeeding | | | | | ANOVA | | |
	0	1	2	3	6	D	T	D × T
Glucose (mmol/l)								
Placebo	8.1 ± 0.1	8.2 ± 0.3	9.3 ± 0.4	8.8 ± 0.3	8.7 ± 0.4			
ACOL	7.5 ± 0.1	8.4 ± 0.2	8.6 ± 0.2	9.1 ± 0.3	8.3 ± 0.2	NS	0.0003	NS
Insulin (nmol/l)								
Placebo	0.16 ± 0.02	0.55 ± 0.01	0.40 ± 0.08	0.42 ± 0.08	0.19 ± 0.05			
ACOL	0.11 ± 0.02	0.29 ± 0.01	0.31 ± 0.03	0.30 ± 0.09	0.14 ± 0.02	0.0008	<0.0001	NS
HOMA-IR (units)								
Placebo	9.9 ± 1.2							
ACOL	5.9 ± 0.8[a]							

HOMA-IR, homeostasis model assessment of insulin resistance. Each point and column represents the average ± SEM of five to six animals. The ANOVA columns depict the level of significance (P) of the main effects of drug (D) and time (T) and of their interaction (D × T) determined by factorial ANOVA.
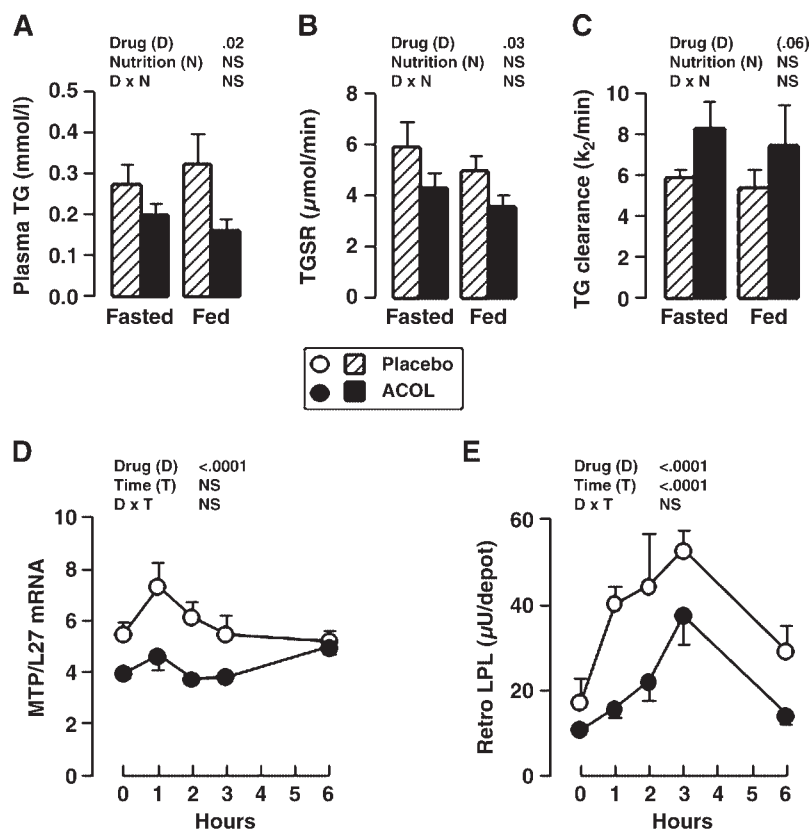[a] Different from placebo ($P < 0.03$ by Student's t-test).

Fig. 1. Serum triglyceride (TG) concentrations (A), triglyceride secretion (TGSR; B), and clearance (C) rates after a 12 h fast and 2 h after refeeding a fat-free meal. Microsomal triglyceride transfer protein (MTP) mRNA levels (D) and retroperitoneal white adipose tissue LPL activity (E) after a 12 h fast (0 h time point) and 1, 2, 3, or 6 h after the onset of a 2 h period of refeeding their habitual diet, in rats treated (black circles and columns) or not (white circles, hatched columns) with acolbifene (ACOL) for 4 weeks. Each point and column represents the average $\pm$ SEM of five to six animals. The ANOVA table that accompanies each panel presents the main and interactive effects of treatment and time as determined by factorial ANOVA.

However, there was a significant decrease with time of refeeding in the HDL fraction of untreated rats (drug $\times$ time interaction). Treatment with ACOL for 4 weeks brought about a robust (-50%) decrease in total CHOL (Fig. 2A), which was as marked in the HDL (Fig. 2B) as in the non-HDL (Fig. 2C) fractions. In contrast with its effect on circulating lipids, chronic ACOL treatment increased liver concentrations of both TG ($+95\%$; Fig. 2D) and CHOL ($+27\%$; Fig. 2E). Of note is the fact that the long-term hypocholesterolemic effect of ACOL was not attributable to lower food intake, as determined in a separate pair-feeding study using an identical treatment design [food intake (g/21 d) of placebo, 313 ± 12; pair-fed placebo, 259 ± 7; ACOL, 253 ± 11; total plasma CHOL (mM) in placebo, 1.8 ± 0.2; pair-fed placebo, 1.9 ± 0.1; ACOL, 1.2 ± 0.1 ($P < 0.004$ with both placebo groups)].

Because ACOL strongly affected cholesterolemia and resulted in the accumulation of both TG and CHOL in the liver, we next addressed the possible involvement therein of key enzymes and receptors that modulate hepatic lipid metabolism. We first determined the expression levels of the SREBP family. SREBP-1a and SREBP-1c are major transcriptional regulators of enzymes of the lipogenic cascade, whereas SREBP-2 is a master regulator of the CHOL synthetic pathway. As shown in **Fig. 3A**, hepatic SREBP-1a mRNA levels were similar in placebo- and ACOL-treated animals in the fasted state and were transiently increased upon refeeding (2- to 3-fold), but significantly more so in ACOL- than in placebo-treated animals. An identical pattern was observed for SREBP-1c mRNA levels (Fig. 3B), whereas those of SREBP-2 remained unaltered by chronic

ACOL treatment and acute refeeding (Fig. 3C). The liver concentration of PPARα protein, which modulates several aspects of liver TG metabolism, including fatty acid oxidation, was affected neither by chronic ACOL treatment nor by acute food intake (data not shown).

The activity of HMG-CoA reductase, the rate-limiting enzyme in de novo CHOL synthesis, was identical in the fasted state in placebo- and ACOL-treated groups (**Fig. 4A**). Refeeding increased enzyme activity more than 2-fold in both groups. In ACOL-treated rats, the postprandial increase in HMG-CoA reductase activity was prolonged to 6 h after the onset of refeeding, whereas it had returned to fasting levels at that time in placebo-treated animals. The activity of liver ACAT, which modulates cytoplasmic free CHOL through its storage in esterified form and can thereby affect both CHOL synthesis and lipoprotein secretion, was identical in both groups in the fasted state (Fig. 4B). Refeeding reduced ACAT activity, but more so in placebo- than in ACOL-treated rats. Finally, the protein levels of two major receptors involved in the hepatic uptake of CHOL from the circulation were quantified. Fasting liver SR-BI protein levels were nearly 3-fold higher in ACOL-treated than in control rats (Fig. 4C). In the fasted state, liver SR-BI levels correlated negatively with plasma HDL-CHOL levels ($r = -0.80$, $P < 0.002$, n = 12). Refeeding increased SR-BI protein in untreated rats to levels found in ACOL-treated rats, which themselves were not altered by refeeding. ACOL treatment resulted in a slight reduction (-14%) in SR-BI mRNA levels [pooled placebo, 1.4 ± 0.1 (n = 26); ACOL, 1.2 ± 0.1 (n = 30) SR-BI/L27 mRNA; $P < 0.04$]. Whereas time after refeeding did not significantly
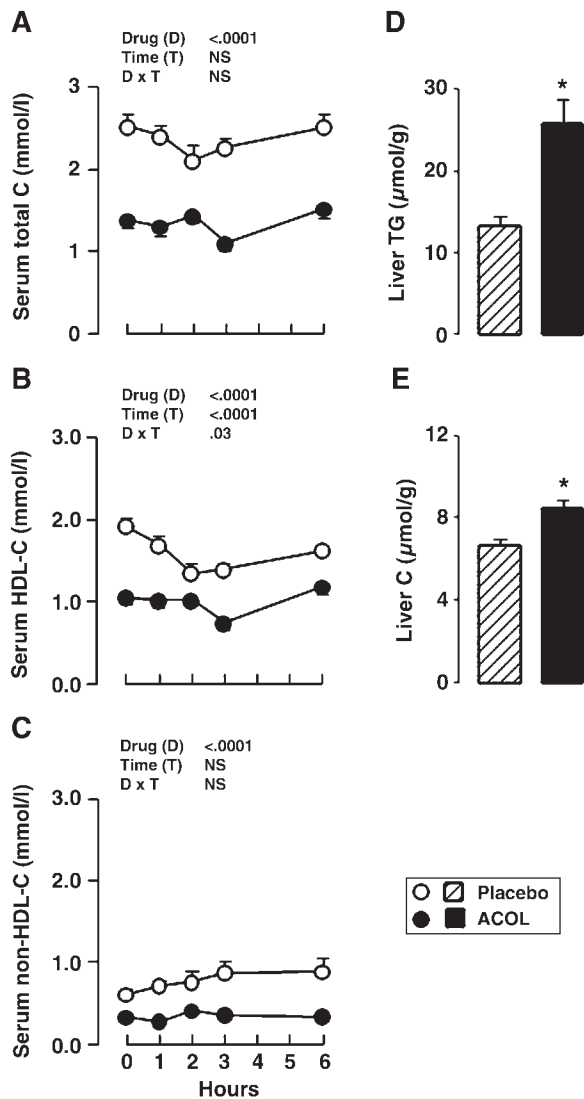
Fig. 2. Serum total (A), HDL (B), and non-HDL (C) cholesterol (C) concentrations after a 12 h fast (0 h time point) and 1, 2, 3, or 6 h after the onset of a 2 h refeeding period in rats treated (black circles) or not (white circles) with ACOL for 4 weeks. Each point represents the average ± SEM of five to six animals. Liver cholesterol (D) and TG (E) concentrations in rats treated (black columns) or not (hatched columns) with ACOL for 4 weeks. Each column represents the average ± SEM of 23–24 animals. * $P \leq 0.0001$. See legend to Figure 1 for description of ANOVA tables.
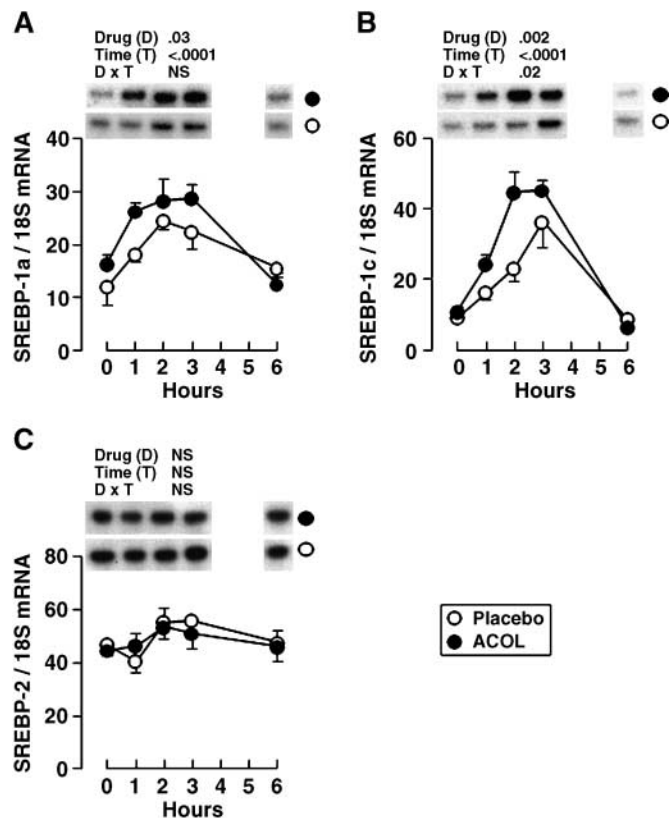

Fig. 3. Liver sterol-regulatory element binding protein-1a (SREBP-1a; A), SREBP-1c (B), and SREBP-2 (C) mRNA concentrations after a 12 h fast (0 h time point) and 1, 2, 3, or 6 h after the onset of a 2 h refeeding period in rats treated (black circles) or not (white circles) with ACOL for 4 weeks. Each point represents the average ± SEM of five to six animals. The gels at top are representative Northern blots of the SREBP mRNA. The reference 18S mRNA (not shown) was identical at all time points and in both treatment groups. See legend to Figure 1 for description of ANOVA tables.

affect liver LDLR protein levels, chronic ACOL increased liver LDLR protein ~2-fold compared with the placebo group (Fig. 4D). LDLr mRNA levels remained unaffected by ACOL [pooled placebo, 4.5 ± 0.4 (n = 26); ACOL, 4.7 ± 0.3 (n = 30) LDLR/L27 mRNA; NS].

DISCUSSION

This study was undertaken to establish the mechanisms by which the SERM ACOL decreases plasma lipids. It was found that triglyceridemia was decreased by ACOL under the present dietary conditions, mainly as a result of a reduced rate of VLDL-TG secretion, which in turn was asso-

ciated with a decrease in MTP mRNA levels. In addition, cholesterolemia was decreased by more than 50%, and both HDL and non-HDL fractions were affected. ACOL exerted its hypocholesterolemic action in the absence of major changes in determinants of CHOL synthesis or storage, suggesting an action upon hepatic uptake of CHOL from serum CHOL-rich lipoprotein particles. In accordance with this concept, chronic treatment with ACOL was associated with a robust increase in the abundance of both the LDLR and SR-BI in the liver.

The present study confirms previous findings regarding the effects of ACOL on body weight, food intake, and fat deposition (9, 10). The decrease in body weight, mainly in the form of fat stores, was associated with a reduction in food intake and food efficiency. Muscle mass accretion was largely maintained in ACOL-treated rats. Therefore, the drug shares some actions on energy and fat metabolism with other antiestrogens (11–15) and estradiol metabolites with low estrogenic activity (40). The present study also extends previous findings on insulin sensitivity (9, 10), which have previously been limited to fasting glucose/insulin levels, by demonstrating that the insulin response to a physiological chal-
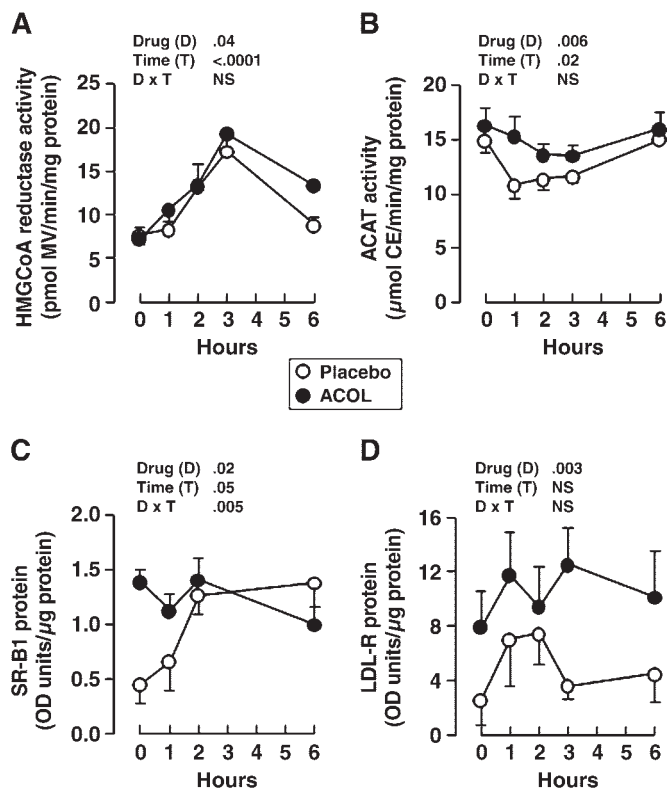
Fig. 4. Liver HMG-CoA reductase (A) and ACAT (B) activities, and scavenger receptor class B type I (SR-BI; C) and LDL receptor (LDLR; D) protein concentrations, after a 12 h fast (0 h time point) and 1, 2, 3, or 6 h after the onset of a 2 h refeeding period in rats treated (black circles) or not (white circles) with ACOL for 4 weeks. CE, cholesteryl ester; MV, mevalonate; OD, optical density. Each point represents the average ± SEM of five to six animals. See legend to Figure 1 for description of ANOVA tables.

lenge (refeeding) is dramatically improved by ACOL. Because of the strong link between adipose tissue and insulin action (41), the ACOL-induced reduction in food intake and the concomitant decrease in fat mass accretion most likely contributed to the improvement in insulin sensitivity.

Variations related to the acute nutritional status in determinants of TG metabolism have been studied extensively, and their modulation during the fasting-feeding transition was largely confirmed in the present study. Several modulators of hepatic CHOL and TG metabolism were also modified by acute food intake in the short term, in agreement with earlier reports, including the refeeding-induced increase in HMG-CoA reductase and decrease in ACAT activities (42–44), the postprandial increase in SREBP-1a and SREBP-1c mRNA levels (45, 46), and the lack of change in SREBP-2 (46). Of note is the novel nutritional modulation of liver SR-BI that was revealed here, as refeeding after a 12 h fast increased liver SR-BI content 3-fold in rats not treated with ACOL. Interestingly, the high fasting SR-BI levels in ACOL-treated rats were not further modulated by food intake. The strong upregulation of SR-BI by ACOL in the fasted state and lack thereof in the fed state have been confirmed in two additional studies (C. Lemieux and Y. Deshaies, unpublished data). The detailed time course

and mechanisms of the feeding-induced increase in SR-BI protein in control rats remain to be determined.

A significant lowering effect of ACOL on VLDL-TG secretion rate and a borderline increase in TG clearance rate were observed in both fasted and fed conditions, resulting in lower triglyceridemia. Estrogen increases serum TG levels in humans and rodents (reviewed in 8) as a result of a direct action of estrogen on the hepatic secretion of TG-rich VLDL (47); therefore, the ability of ACOL to reduce VLDL-TG secretion is of particular interest. The ACOL-induced decrease of MTP mRNA levels suggests that the steroid interfered with VLDL assembly and secretion (48). Whether this effect results from a direct action of ACOL or is an indirect consequence of improved insulin sensitivity (49) remains to be determined. Of relevance to this issue is the fact that a direct action of phytoestrogens on MTP expression and activity has been demonstrated (50). The increased abundance of the LDLR (discussed below) also could have contributed to lower net VLDL secretion by favoring VLDL reuptake. With regard to TG clearance rate, it tended to be increased by ACOL despite a large reduction in the global availability of adipose LPL. It should be mentioned that ACOL has been found to favor nitric oxide-mediated vasodilation (51, 52), suggesting that the LPL-independent improvement in TG clearance may be attributable to vascular effects resulting in the increased delivery of TG-rich lipoproteins to LPL-rich capillary beds. The fact that ACOL did not mimic the hypertriglyceridemic action of estrogen is of particular significance in view of the potentially deleterious consequences of high TG levels on cardiovascular risk, particularly in women (2).

ACOL increased liver TG content, which is at variance with the expected decrease of liver lipids after a reduction in energy intake. This implies that ACOL acted directly upon liver TG metabolism, by increasing hepatic TG synthesis, reducing disposal through fatty acid oxidation, or favoring retention by virtue of a reduced rate of lipoprotein secretion. In the present study, plasma nonesterified fatty acid levels (precursors for lipogenesis) were unchanged by ACOL (data not shown). However, the amplification of the transient postprandial increase in SREBP-1a and SREBP-1c by ACOL may have favored postprandial hepatic lipogenesis. Hepatic retention of TG as a result of decreased MTP expression and VLDL-TG secretion (in both the fasted and fed states) are also likely contributors to the increase in liver TG content. Finally, it should be noted that the latter (from 1.0 to 2.5%, w/w) did not reach levels considered steatotic according to human criteria [>5–10% (53)].

The ACOL-induced decrease of plasma CHOL concentrations occurred in rats consuming a CHOL-free diet and therefore was independent of CHOL intake. The decrease was observed in both the HDL and non-HDL fractions. Because HDL constitutes more than two-thirds of total CHOL in the rat, its decrease explained most of the hypocholesterolemic effect of ACOL. De novo CHOL synthesis did not appear to play a major role in the hypocholesterolemic action of ACOL because both SREBP-2 mRNA level and HMG-CoA reductase activity were not much affected by ACOL. Interestingly, the ACOL-induced in-

crease in liver CHOL content, which occurred in the free CHOL fraction only (C. Lemieux and Y. Deshaies, unpublished data) and to which the blunted postprandial ACAT reduction may have contributed, proved to be insufficient to modulate HMG-CoA reductase activity. A decrease in CHOL export from the liver to the circulation may have been involved in the serum CHOL-lowering effect of ACOL, as suggested by the reduction in VLDL secretion. However, the magnitude of the hypocholesterolemic effect of ACOL, its extension to all lipoprotein fractions, and the robust impact of ACOL on CHOL-rich lipoprotein receptors strongly suggest the involvement of hepatic CHOL uptake from the circulation.

Estrogen affects cholesterolemia in a dose-dependent manner in rodents. At physiological levels, estrogen increases HDL-CHOL through enhanced hepatic apolipoprotein A-I synthesis, but as doses reach the pharmacological range, LDLR expression is induced (54–56), with a resulting decrease in both LDL- and HDL-CHOL (21). High-dose estrogen also results in a large reduction in SR-BI abundance in the liver, along with a dramatic SR-BI increase in steroidogenic cells of the adrenal gland and ovary (29, 30, 57). The present study demonstrates that ACOL exerts an estrogen-like effect on hepatic LDLR abundance, with a 2-fold increase in protein levels after 4 weeks of treatment. A lack of change in mRNA levels suggests posttranscriptional mechanisms. The LDLR is well known to be regulated largely at the level of gene expression (58); however, evidence for posttranscriptional regulation by estrogen, statins, and dietary CHOL has been reported (29, 59–62). Remarkably, in contrast with the effects of estrogen, ACOL increased liver SR-BI protein, apparently by counteracting the large reduction in hepatic receptor levels induced by short-term fasting that was observed in untreated rats. The mechanisms whereby steroid hormones, and ACOL in particular, affect SR-BI remain to be fully established. As in the case of the LDLR, SR-BI mRNA was not upregulated by ACOL, suggesting posttranscriptional pathways. It has been suggested that the estrogen-induced reduction in liver SR-BI is indirect and dependent upon the upregulation of the LDLR (30). Clearly, such an inverse relationship between the two receptors did not exist under ACOL treatment. Additional studies are needed to determine whether ACOL affects SR-BI directly or indirectly and to identify the specific posttranscriptional steps in SR-BI metabolism that are targeted by the steroid.

The contributions of the LDLR and SR-BI to the CHOL-lowering action of ACOL, which are suggested by the present study, remain to be established. As noted above, high-dose estrogen decreases HDL-CHOL in the rat despite a large reduction in liver SR-BI, which may be compensated for by its robust increase in steroidogenic tissues with high HDL uptake activity (29, 30, 57). Increased liver LDLR may explain at least part of the lowering effect of ACOL on CHOL transported by the non-HDL fraction and apolipoprotein E-containing HDL (63). In the rat, however, the liver removes 60–70% of HDL cholesteryl esters from plasma via the selective uptake (non-LDLR) pathway (64). Because of the relatively low abundance of apoli-

poprotein E-HDL in the rat (65), its uptake by the LDLR should represent only a fraction of total HDL-CHOL clearance. The fact that in the fasted state liver SR-BI protein levels were almost 3-fold higher in ACOL-treated than in control rats suggests that selective uptake of HDL cholesteryl ester may have been increased by ACOL. Another argument in favor of the involvement of liver SR-BI is the fact that it is only in untreated rats that, upon refeeding, both a decrease in HDL-C and an increase in SR-BI occurred concomitantly. In ACOL-treated rats, acute refeeding elicited changes neither in HDL-CHOL nor in SR-BI, thus supporting a contribution of SR-BI to the determination of plasma HDL-CHOL. Such a relationship is further suggested by the strong negative correlation noted between liver SR-BI and plasma HDL-CHOL in fasted rats.

The consequences of the HDL-CHOL-lowering action of ACOL in rats must be considered in the context of the fundamental differences that exist between human and rodent lipoprotein metabolism. As alluded to above, most CHOL is transported by HDL in the rat, and a robust lowering of total CHOL seldom occurs without a decrease in this fraction. Based upon gene manipulation studies, the present knowledge suggests clear beneficial consequences of high SR-BI and reverse CHOL transport activities (24–26). Therefore, the eventual confirmation in humans of the ACOL-mediated increase in liver SR-BI abundance, along with the increase in the LDLR, would both be considered positive effects in terms of the cardiovascular risk associated with lipoprotein metabolism.

In conclusion, ACOL exerted an action opposite to that of estrogen in reducing triglyceridemia and the rate of hepatic VLDL-TG secretion. This action was associated with a reduction in MTP mRNA levels. In addition, the SERM exerted a marked hypocholesterolemic action independent of changes in major determinants of CHOL synthesis; instead, it increased the abundance of the LDLR and SR-BI in the liver, which suggests an increase in hepatic uptake of CHOL from CHOL-rich lipoprotein particles. Therefore, in addition to its anticarcinogenic, antiobesity, insulin-sensitizing, and bone-mineralization actions, ACOL beneficially modulates TG metabolism while exerting the action of a potent hypocholesterolemic agent. ACOL acts upon hepatic CHOL metabolism through pathways that are entirely different from those of conventional hypocholesterolemic drugs, and it distinguishes itself from natural estrogen in that it positively modulates the SR-BI in addition to the LDLR. ■

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