Available online at www.sciencedirect.com

SCIENCE DIRECT®

Metabolism Clinical and Experimental 55 (2006) 605–613

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Hypocholesterolemic action of the selective estrogen receptor modulator acolbifene in intact and ovariectomized rats with diet-induced hypercholesterolemia

Christian Lemieux[a], Yves Gélinas[a], Josée Lalonde[a], Fernand Labrie[b], Denis Richard[a], Yves Deshaies[a,*]

[a]*Laval Hospital Research Center, Department of Anatomy and Physiology, Faculty of Medicine, Laval University, Quebec City, Quebec, Canada G1K 7P4*
[b]*Molecular Endocrinology and Oncology Research Center of Laval University Hospital Center, Department of Anatomy and Physiology, Faculty of Medicine, Laval University, Quebec City, Quebec, Canada G1K 7P4*

Received 9 September 2005; accepted 18 November 2005

Abstract

Acolbifene (ACOL) is a fourth-generation selective estrogen receptor modulator (SERM) that has strong and pure antiestrogenic properties toward estrogen-sensitive cancers, but improves energy and lipid metabolism in an estrogen-like fashion in rodent models. The aim of this study was to determine the potency of ACOL to reduce cholesterolemia in a dietary model of hypercholesterolemia and to establish its mechanisms of action. Intact and ovariectomized (OVX) female rats were treated for 3 weeks with ACOL, and serum cholesterol and liver determinants of cholesterol metabolism were assessed. Acolbifene prevented both diet- and ovariectomy-induced weight gain and completely prevented diet-induced hypercholesterolemia. Relative to a reference chow diet, the high-cholesterol diet decreased the high-density lipoprotein (HDL) cholesterol fraction, which remained unaffected by ACOL, indicating that in hypercholesterolemic conditions, ACOL modulated only the non-HDL fraction. No impact of ACOL on determinants of liver cholesterol synthesis was observed. In contrast, ACOL increased hepatic low-density lipoprotein receptor protein in both intact and OVX rats, which was negatively correlated with serum total and non–HDL cholesterol ($r = -0.59$, $P < .0001$), suggesting a contribution of receptor-mediated hepatic uptake of cholesterol-rich lipoproteins to the hypocholesterolemic effect of ACOL. These findings establish that ACOL retains its powerful cholesterol-lowering action in diet-induced hypercholesterolemia and suggest that the SERM acts in such conditions through favoring hepatic low-density lipoprotein receptor–mediated uptake of cholesterol transported by non-HDL lipoprotein fractions.

1. Introduction

Hypercholesterolemia is one of the major risk factors for coronary heart disease [1], and lowering plasma cholesterol decreases such risk in proportion to the extent of cholesterol lowering [2]. Cholesterolemia is of relevance to the menopausal transition, during which the proportion of cholesterol transported by low-density lipoprotein (LDL) increases at the expense of high-density lipoprotein (HDL) [3-5].

In previous studies [6-8], we and others have demonstrated positive effects on lipid metabolism of acolbifene (ACOL), a fourth-generation selective estrogen receptor modulator (SERM). The compound has been developed to prevent and treat estrogen-sensitive cancers, and it might also prove useful in the prevention of the metabolic deterioration associated with menopause [9]. Acolbifene was thus shown to exert a powerful cholesterol-lowering action in various rat and mouse models [6-8,10], independently of its effect on food intake [11]. A previous study [10] has suggested that this is at least partly achieved through liver up-regulation of the LDL receptor (LDLr), a proestrogenic action [12-15], as well as that of the scavenger receptor, class B, type 1 (SR-B1), an antiestrogenic action [16], both receptors favoring hepatic cholesterol clearance from various lipoprotein fractions.

Given that current lifestyle trends favor not only obesity and insulin resistance but also hypercholesterolemia, and that women in the menopause transition are particularly vulnerable to the appearance of these abnormalities, it appeared relevant to assess the potency of the SERM ACOL in preventing diet-induced hypercholesterolemia. Intact and

* Corresponding author. Tel.: +1 418 656 3705; fax: +1 418 656 7898.
E-mail address: yves.deshaies@phs.ulaval.ca (Y. Deshaies).

0026-0495/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2005.11.016

ovariectomized (OVX) female rats were studied to include models of both pre- and postmenopausal conditions. Because of the shift in cholesterol distribution toward non-HDL lipoproteins brought about by cholesterol feeding [17,18], particular attention was given to treatment effects on the LDLr and its modulators. Dietary cholesterol decreases the abundance of the LDLr in the liver [19] consequent to an increase in intrahepatocyte cholesterol concentration. The latter in turn decreases sterol regulatory element binding proteins (SREBPs), which are master modulators of the expression of the LDLr and several lipogenic genes [20,21]. Such modulation of the LDLr by dietary cholesterol is therefore diametrically opposed to that exerted by ACOL in the rat. The LDLr as well as other key modulators of hepatic cholesterol metabolism, including those of sterol synthesis and excretion, were therefore assessed with the goal of identifying the levels of interaction of ACOL and cholesterol feeding, as well as the mechanisms of action of ACOL in hypercholesterolemic conditions.

2. Methods

2.1. Animals and treatments

Twenty-nine female Sprague-Dawley rats initially weighing 175 to 200 g were purchased from Charles River Laboratories (St-Constant, Quebec, Canada) and housed individually in stainless steel cages in a room kept at 23°C ± 1°C with a 12-hour light-dark cycle (lights on at 7:00 PM). The animals were cared for and handled in conformity with the Canadian Guide for the Care and Use of Laboratory Animals, and the protocol was 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 to a nonpurified rodent diet (Charles River Rodent Diet no. 5075, Ralston Products, WoodStock, Ontario, Canada), with an energy density of 12.9 kJ/g. Five rats were fed with the chow diet throughout the experimental period and were not subjected to any manipulation or treatment. This group served as a reference to verify the metabolic effects of the high-cholesterol diet. The remaining 24 rats were switched to a purified diet enriched with 2% (wt/wt) cholesterol (C diet) that provided 50% energy as carbohydrate, 30% as fat, and 20% as protein, with an energy density of 18.0 kJ/g. The diet was designed to mimic the typical western diet by providing refined carbohydrates (sucrose) and saturated fat (beef tallow), in addition to high levels of dietary cholesterol, to maximize the dietary challenge against which to assess the potency of ACOL. The composition of the diet is described elsewhere [11]. Half of the 24 rats were assigned to the control group and the 12 remaining animals were treated with ACOL {2*H*-1-benzopyran-7-ol,3-(4-hydroxyphenyl)-4-methyl-2-[4-[2-(1-piperidinyl)ethoxy]phenyl]–, hydrochloride, (2*S*)–; also known as SCH57068 · HCl or EM-652 · HCl}, which was given as previously reported [8], that is, once daily by oral gavage at a dose of 2.5 mg/kg in a total volume of 0.5 mL of an aqueous solution of 0.4% methylcellulose. The control group received the vehicle alone. At the onset of feeding the purified diet, 6 rats from the control group and 6 from the ACOL group were OVX under isoflurane anesthesia. The animals of the intact group were subjected to the same surgical procedure except that the ovaries were not excised. Four groups were therefore formed according to a 2 × 2 factorial design. Treatment was provided for a period of 21 days. Food intake and body weight were monitored every other day. Rats were studied in the postprandial state after refeeding for a specific period to ensure that measurements would be made in the short-term presence of dietary cholesterol and under well-defined nutritional conditions. To this end, the day before the completion of the study, food was removed after the last gavage at 9:00 PM, that is, at the onset of the lighted period during which food intake is considerably reduced in ad libitum conditions. The following day, food was returned to cages at the beginning of the dark period between 7:00 and 9:00 AM, and rats were killed by decapitation exactly 2 hours later. A corollary study was performed under the above treatment conditions, except that rats were not refed on the last day of treatment and were killed in the fasted state. Liver samples were used to measure LDLr protein as described below.

2.2. Blood and tissue collection

Blood was collected from the neck wound and immediately centrifuged at 1500g, 4°C, for 15 minutes. Serum was stored at −80°C for later lipid measurements. A sample of liver was immediately frozen in liquid nitrogen and stored at −80°C until later lipid and protein determinations. Retroperitoneal and inguinal white adipose tissues, and vastus lateralis and soleus muscles were excised and weighed.

2.3. Serum/tissue lipid measurements

Serum glucose concentration was measured by the glucose oxidase method using the YSI Stat glucose analyzer (YSI, Yellow Springs, OH). Serum insulin was determined by radioimmunoassay using a reagent kit from Linco Research (St Charles, MO) with rat insulin as standard. Serum cholesterol was quantified in total serum and in the HDL fraction, isolated by precipitation of apolipoprotein B (apoB)–containing lipoproteins with Na-phosphotungstate-$MgCl_2$, using a reagent kit from Wako Diagnostics (Richmond, VA). Frozen liver samples were thawed, and total lipids were extracted according to the method of Folch et al [22] and solubilized in isopropanol. Liver total and free (unesterified) cholesterol were quantified in the lipid extracts using reagent kits from Wako Diagnostics. Liver esterified cholesterol was obtained by difference.

2.4. Immunoassay of the LDLr

The LDLr protein was extracted and quantified using an anti-LDLr antibody kindly provided by Dr Joachim Herz (South Western Medical Center, Dallas, TX), as previously

described [10]. Protein concentration of the liver extracts was determined by the method of Lowry et al [23], and protein separation by electrophoresis was performed according to the method of Laemmli [24].

2.5. RNAse protection assay of SREBP-1a, SREBP-1c, and SREBP-2 messenger RNA

The SREBP messenger RNA (mRNA) transcripts were quantified by RNAse protection assays as previously described [10]. The complementary DNA (cDNA) probe for rat SREBP-1a, SREBP-1c, and SREBP-2 was generated as described by Shimomura et al [25]. The cDNA fragment for rat SREBP-1a and SREBP-1c was amplified by reverse transcriptase–polymerase chain reaction (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′-3′) ATG GAC GAG CTG CCC TTC GGT GAG GCG GCT and 3′primer (5′-3′) CCA GAG AGG AAC CCA GGG AAG CAG. 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′-3′) GAG CTG ACT CTC GGG GAC AT and 3′primer (5′-3′) ACT GCC GCC ACC ACC TCC AG. The cDNA fragment was subcloned into the pGEM-T vector. The protected fragments corresponding to rat SREBP-1a (257 base pairs [bp]), SREBP-1c (150 bp), and SREBP-2 (120 bp) were quantified with the BioRad Imaging Densitometer (Mississauga, Ontario, Canada). 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.

2.6. Liver RNA isolation and analysis of LDLr, ATP binding cassette G5, ATP binding cassette G8, and 7α-hydroxylase mRNA

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 the Expand reverse transcriptase (Roche Diagnostics, Laval, Quebec, Canada). The expression level of mRNA was assessed using quantitative fluorescent real-time PCR (Corbett Research, New South Wales, Australia). Amplification and detection of target mRNAs were performed with Platinum Taq polymerase and the intercalating dye Sybr-Green I. The primers, designed using the Vector NTI program (Informax, Frederick, MD) and synthesized by Invitrogen (Burlington, Ontario, Canada), were the following: for LDLr, 5′primer (5′-3′) TGG ACC CTT TCT CTC GGA AC, 3′primer (5′-3′) AAG GCT GTG GGT TCC ATA GG; for ATP binding cassette (ABC) G5, 5′primer (5′-3′) TCG GGC CCT GGT GGA ACA TCA A, 3′primer (5′-3′) AGC CAG CAT CGC CGT GTA TCT CAG; for ABCG8, 5′ primer (5′-3′) AAC AGC CTC TAC TTC ACC TA, 3′primer (5′-3′) TCT CTG ACA GTC AGA TTG GG; for 7α-hydroxylase (CYP7A), 5′primer (5′-3′) CCG TCT ACG TAT GTT TCT CA, 3′primer (5′-3′) AGA TGG AGA GTG AAG TCC TC; and for L27, 5′primer (5′-3′) CTG CTC GCT GTC GAA ATG, 3′ primer (5′-3′) CCT TGC GTT TCA GTG CTG. The mRNA levels of all 4 genes 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.

2.7. 3-Hydroxy-3-methylglutaryl coenzyme A reductase and acyl coenzyme A:cholesterol acyltransferase activities

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was quantified by measuring mevalonolactone formation from HMG-CoA and that of acyl coenzyme A:cholesterol acyltransferase (ACAT) by the esterification of free cholesterol with labeled oleate as previously described [10].

2.8. Statistical analysis

Data are expressed as means ± SEM. The effect of the high-cholesterol diet on serum cholesterol fractions, liver cholesterol concentration, and HMG-CoA reductase activity was tested against the reference chow diet by Student unpaired t test. Within the cohort fed with the high-cholesterol diet, main and interactive treatment effects were analyzed by a 2 × 2 factorial analysis of variance (ANOVA), the factors being surgery with 2 levels (intact, OVX) and drug treatment with 2 levels (control, ACOL). Post hoc pairwise comparisons of group means were made by Fisher protected least significant difference test to locate individual between-group differences.

3. Results

The metabolic effects of the high-cholesterol diet relative to the reference chow diet are summarized in Table 1, which compares and contrasts the effect of diet alone in untreated rats. Feeding the C diet resulted in greater body weight gain and adipose mass, which were strongly correlated with each other ($r = 0.77$, $P < .0001$), and the higher weight gain was associated with greater cumulative energy intake ($r = 0.88$, $P < .0001$). As expected, total serum cholesterol was increased nearly 4-fold by the C diet, which also profoundly altered cholesterol distribution among lipoprotein fractions. Indeed, whereas HDL transported 65% of total cholesterol in chow-fed animals, this proportion fell to 6% in C diet–fed rats, with a concomitant 64% decrease in absolute HDL cholesterol (HDL-C) levels compared with chow-fed rats. Liver cholesterol concentration was elevated 10-fold by the C diet. Both nonesterified (free) and esterified cholesterol concentrations were increased, the latter becoming a much larger proportion of liver cholesterol than in chow-fed rats. High liver cholesterol was associated with the expected down-regulation of HMG-CoA reductase activity, which fell to one third of that in the liver of chow-fed animals. The C diet therefore exerted its intended actions on cholesterol metabolism.

Table 1
Effect of the purified, cholesterol-enriched diet relative to the reference chow diet on body weight gain, sum of inguinal and retroperitoneal white adipose depots, cumulative energy intake, postprandial serum total and lipoprotein cholesterol concentrations, liver total, free, and esterified cholesterol concentrations, and liver HMG-CoA reductase activity

	Reference (chow)	Purified + 2% cholesterol
Body weight gain (g)	42 ± 6	76 ± 9*
Sum adipose tissue weights (g)	2.3 ± 0.2	4.2 ± 0.3*
Cumulative energy intake (MJ)[a]	5.3 ± 0.3	7.2 ± 0.3*
Total cholesterol (mmol/L)	1.7 ± 0.2	6.4 ± 0.7*
HDL-C (mmol/L)	1.1 ± 0.2	0.4 ± 0.1*
Non–HDL-C (mmol/L)	0.6 ± 0.1	5.9 ± 0.7*
Liver total cholesterol (μmol/g)	6.0 ± 0.1	59.8 ± 4.9*
Free	5.2 ± 0.1	22.3 ± 4.4*
Esterified	0.8 ± 0.1	37.6 ± 1.9*
HMG-CoA reductase activity	104 ± 14	35 ± 7*

Values are means ± SEM of 5 to 6 animals. HMG-CoA reductase activity is expressed in pmol mevalonate/min per milligram of protein.

[a] Energy intake was calculated from food intake and the respective energy density of each of the 2 diets.

* $P < .05$, different from reference diet (unpaired Student t test).

All the subsequent data are those obtained in animals fed with the high-cholesterol diet (C diet). The effects of OVX and ACOL treatment on metabolic variables in rats fed with the C diet are presented in Table 2. Ovariectomy nearly doubled body weight gain over the 3-week treatment period, the increase being associated with gain in both fat (inguinal and retroperitoneal depots) and muscle mass (significant for vastus lateralis), confirming the well-known effect of OVX on energy balance. Also confirming previous studies using low-fat, cholesterol-free diets, ACOL decreased weight gain, mostly through a reduction in fat accretion. The drug was as effective in OVX as in intact rats. When considering all groups together, body weight gain and adipose depot weight were closely correlated ($r = 0.77$, $P < .0001$), as were weight gain and food intake ($r = 0.88$, $P < .0001$). Notably, OVX animals ingested significantly more food during the last 2-hour refeeding period than their intact counterparts, regardless of drug treatment.

Treatment effects on postprandial serum total and lipoprotein cholesterol are depicted in Fig. 1. Total cholesterolemia was lower in untreated OVX than in intact rats (Fig. 1A). Long-term ACOL treatment markedly reduced total cholesterol, by two thirds in intact rats and by half in OVX rats. High-density lipoprotein cholesterol levels were slightly but significantly higher in untreated OVX than in intact rats, whereas ACOL maintained HDL-C at a slightly higher level in intact rats compared with their untreated counterparts (Fig. 1B). As evident in Fig. 1C, however, the bulk of treatment effects on cholesterolemia were exerted at the level of the non-HDL fraction, which accounted for all of the OVX- and ACOL-induced decrease in total cholesterol.

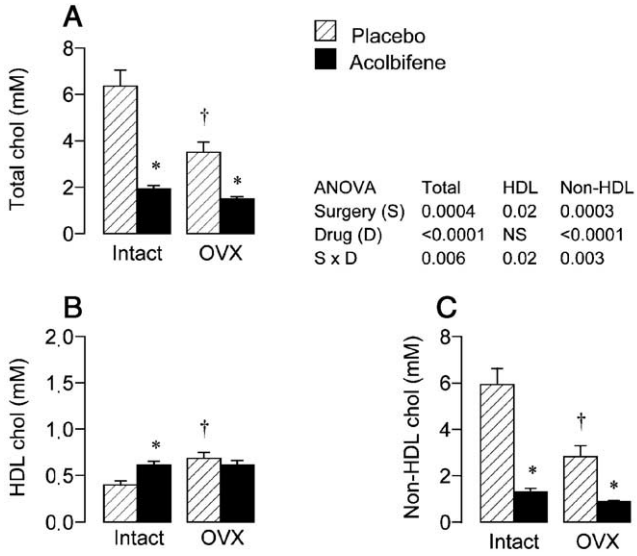

Fig. 1. Serum total (A), HDL (B), and non–HDL-C (C) concentrations in intact and OVX rats treated or not with ACOL for 3 weeks. Rats were studied after a 2-hour refeeding period that followed a 10- to 12-hour fast. Bars represent means ± SEM of 5 to 6 animals. *$P < .05$, different from the placebo group within the same surgery; †$P < .05$, different from the intact group within the same drug treatment. See footnote to Table 2 for significance of the ANOVA table (inset).

As shown in Fig. 2A, the C diet–induced cholesterol accumulation in the liver was identical in intact and OVX animals, as was its distribution between free (Fig. 2B) and esterified forms (Fig. 2C). In both intact and OVX animals,

Table 2
Weight gain, cumulative and terminal food intake, and tissue weights in intact and OVX rats fed with a purified, cholesterol-enriched diet and treated or not with ACOL for 3 weeks

	Intact		OVX		ANOVA		
	Control	ACOL	Control	ACOL	Surgery	Drug	S × D
Weight gain (g)	76 ± 9	35 ± 4*	125 ± 6**	70 ± 6*,**	<0.0001	<0.0001	NS
Cumulative food intake (g)	400 ±15	340 ± 8*	461 ± 16**	398 ± 19*,**	0.0007	0.0006	NS
2-h food intake (g)	5.2 ± 0.6	6.3 ± 0.8	12.1 ± 1.8**	9.3 ± 1.0**	0.0002	NS	NS
Inguinal WAT (g)	2.0 ± 0.2	1.1 ± 0.2*	2.5 ± 0.2	1.4 ± 0.1*	0.04	<0.0001	NS
Retroperitoneal WAT (g)	2.2 ± 0.1	1.3 ± 0.2*	3.0 ± 0.4**	1.5 ± 0.2*	0.05	<0.0001	NS
Vastus lateralis (g)	0.98 ± 0.04	1.01 ± 0.06	1.25 ± 0.05**	1.17 ± 0.06**	0.0005	NS	NS
Soleus (mg)	131 ± 5	119 ± 6	141 ± 9	127 ± 4	NS	NS	NS

Values are means ± SEM of 6 animals. The "ANOVA" column depicts the main and interactive effects of surgery (S) with 2 levels (intact, OVX) and drug treatment (D) with 2 levels (control, ACOL) and their interaction (S × D). WAT indicates white adipose tissue.

* $P < .05$, different from control group within the same surgery.

** $P < .05$, different from intact group within the same drug treatment.

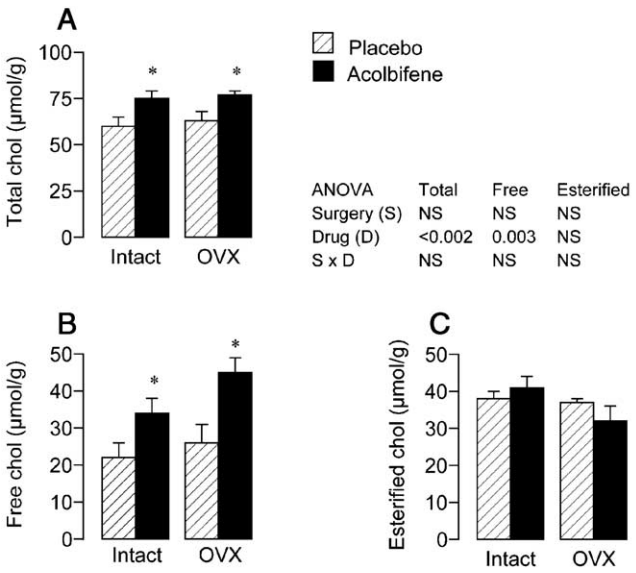

Fig. 2. Liver total (A), free (B), and esterified (C) cholesterol (chol) concentrations in intact and OVX rats treated or not with ACOL for 3 weeks. Bars represent means ± SEM of 6 animals. *$P < .05$, different from the placebo group within the same surgery. See footnote to Table 2 for significance of the ANOVA table (inset).

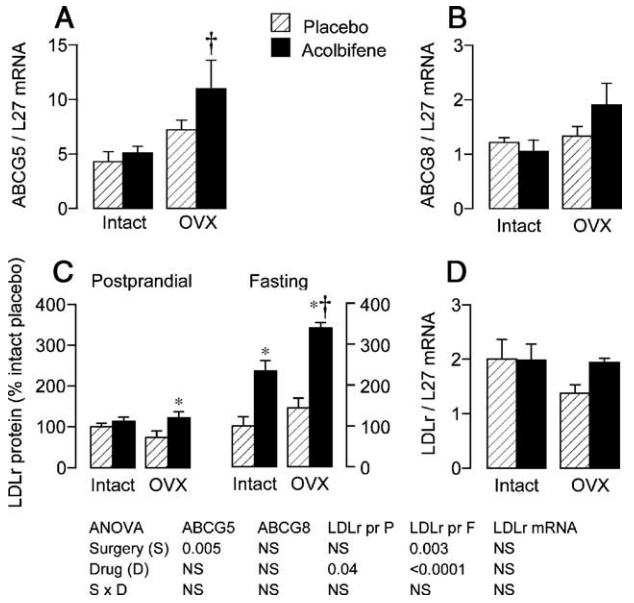

Fig. 3. Liver ABCG5 (A) and ABCG8 (B) relative mRNA levels, LDLr protein concentration (C), and LDLr relative mRNA levels (D) in intact and OVX rats treated or not with ACOL for 3 weeks. Low-density lipoprotein protein was also measured in an additional cohort of animals from which the liver was harvested in the fasting state (F). Low-density lipoprotein receptor protein values (originally in optical density units [OD]/μg protein) were normalized to the mean of the intact placebo group of the respective cohort and are expressed as percentage of the latter. Bars represent means ± SEM of 5 to 6 animals. *$P < .05$, different from the placebo group within the same surgery; †$P < .05$, different from the intact group within the same drug treatment. See footnote to Table 2 for significance of the ANOVA table (inset).

ACOL treatment brought about a small, further increase in liver cholesterol concentration that was entirely due to an elevation in free cholesterol.

The activities or mRNA levels of key determinants of hepatic cholesterol enzymes and proteins are presented in Table 3. The activity of the rate-limiting enzyme in de novo cholesterol synthesis, HMG-CoA reductase, and that of ACAT, which esterifies intracellular cholesterol for storage and therefore impacts cytosolic levels of free cholesterol, were influenced neither by OVX nor by ACOL treatment. The relative mRNA abundance of CYP7A, the rate-limiting enzyme in the synthesis of bile acids from cholesterol, was unaffected by treatment, although it tended to be decreased by ACOL ($P = .07$), especially in OVX rats. These data suggest that cholesterol synthesis, storage, and processing to bile acids were not involved in the changes in serum and liver cholesterol levels elicited by OVX and ACOL. Accordingly, the relative abundance of SREBP-1a, SREBP-1c, and SREBP-2, which are master positive regulators of hepatic lipid metabolism, were not altered by OVX or ACOL treatment.

To investigate whether dietary cholesterol, OVX, and ACOL treatment might have impacted liver cholesterol excretion, we assessed the relative mRNA levels of ABCG5 (Fig. 3A) and ABCG8 (Fig. 3B), 2 membrane transporters that contribute to the biliary excretion of several molecules, including cholesterol. Removal of the ovaries had an overall increasing effect on ABCG5 mRNA levels, whereas ACOL further increased the latter in OVX rats only, such that mRNA of the transporter was higher in ACOL-treated OVX than in

Table 3
Liver HMG-CoA reductase and ACAT activities, and relative mRNA abundance of CYP7A, SREBP-1a, SREBP-1c, and SREBP-2 in intact and OVX rats fed with a purified, cholesterol-enriched diet and treated or not with ACOL for 3 weeks

	Intact		OVX		ANOVA		
	Control	ACOL	Control	ACOL	Surgery	Drug	S × D
HMG-CoA reductase	35 ± 7	34 ± 6	44 ± 5	42 ± 5	NS	NS	NS
ACAT	39 ± 5	44 ± 10	43 ± 9	37 ± 8	NS	NS	NS
CYP7A	6.4 ± 1.2	5.9 ± 0.9	9.2 ± 1.0	6.1 ± 0.6	NS	(0.07)	NS
SREBP-1a	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	NS	NS	NS
SREBP-1c	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	NS	NS	NS
SREBP-2	0.54 ± 0.05	0.50 ± 0.05	0.46 ± 0.05	0.46 ± 0.01	NS	NS	NS

Values are means ± SEM of 4 to 6 animals. CYP7A and SREBP mRNA levels are expressed relative to L27 mRNA. HMG-CoA reductase activity is expressed in pmol mevalonate/min per milligram of protein; ACAT activity is expressed in μmol cholesterol ester/min per milligram of protein. See footnote to Table 2 for significance of "ANOVA" column.

intact treated rats. Expression levels of ABCG8 displayed similar but nonsignificant trends in response to treatment.

Finally, the strong ACOL-induced reduction in serum non–HDL-C concentration prompted us to address the possible involvement of hepatic receptor-mediated cholesterol uptake therein. The effects of surgery and long-term ACOL treatment on protein concentration and mRNA relative abundance of the LDLr are depicted in Fig. 3C and D. When the postprandial cohort of animals was considered, ACOL exerted a significant overall effect on LDLr protein concentration (Fig. 3C, $P = .04$). The latter was clearly increased by ACOL in OVX rats, but only tended to be so in intact animals. To extend the database on treatment effects, LDLr protein was measured in liver homogenates obtained in an identical study, but in which livers were harvested from rats subjected to a 12-hour fast without subsequent refeeding. In these fasted animals, ACOL also robustly increased LDLr protein in OVX rats, and the trend toward an increase observed in intact refed rats became clearly significant. Notably, in the refed cohort, the increase in LDLr protein concentration was not paralleled by changes in LDLr mRNA levels (Fig. 3D). Both serum total and non–HDL-C concentrations, but not that of HDL-C, were negatively correlated with liver LDLr protein concentration ($r = -0.59$, $P < .0001$).

4. Discussion

The present study aimed to assess the potency of the SERM ACOL to reduce cholesterolemia in intact and OVX rats with diet-induced hypercholesterolemia and to investigate its mechanisms of action under such conditions. The findings demonstrate that ACOL totally blunted the diet-induced increase in non–HDL-C levels, both in the presence and absence of endogenous estrogen. In addition, the study suggests that the up-regulation of hepatic LDLr abundance contributed to the robust hypocholesterolemic action of ACOL.

The study confirms the well-known deleterious metabolic effects of the sucrose- and fat-containing diet, as well as the obesity-promoting effect of ovariectomy [26-31]. As shown previously [7,8], treatment with ACOL reduced weight (mostly fat) gain in both intact and OVX animals, largely through a reduction in food intake.

As expected in rats, dietary cholesterol greatly increased serum non–HDL-C and decreased HDL-C. In this species, more than two thirds of cholesterol is normally transported by HDL [32-35], whereas very low-density lipoprotein (VLDL), intermediate-density lipoprotein, and LDL are in very low concentrations, such proportions being inverted by dietary cholesterol [33-35]. The inflow of cholesterol into hepatocytes induces within hours apoB production and the subsequent secretion of apoB-containing, cholesterol-enriched VLDL into the circulation, which are then partly converted to intermediate-density lipoprotein and LDL [17,18,36,37]. All these particles, including VLDL after some delipidation (remnants), are largely cleared from the circulation by the LDLr. It should be noted that in the present postprandial conditions, the non-HDL fraction also included chylomicron-bound cholesterol of dietary origin.

Intriguingly, estrogen removal was associated with a decrease in serum cholesterol relative to intact animals despite the fact that OVX rats ingested more food during the terminal refeeding period than intact rats. In humans, estrogen is considered to be hypocholesterolemic [38,39], whereas in the rat, the hormone impacts HDL at physiologic doses and reduces non–HDL-C (through an increase in the LDLr) at pharmacologic doses [12,14,40,41]. Studies on the effect of ovariectomy on cholesterolemia in rodents, including ours, do not reach a consensus, alternatively reporting an absence of effect [8,42,43] or increased cholesterolemia [7,44,45]. The impact of OVX on cholesterolemia in the presence of dietary cholesterol is even less well established [46]. Because estrogen shares common targets with several cholesterol-modulating nutrients, OVX may conceivably exert different actions on cholesterol metabolism depending on the nature of the diet, and further investigation specifically aimed at this issue is clearly needed.

The modest ACOL-induced increase in liver cholesterol content confirms previous findings [8]. The present study extends these findings by demonstrating that it is the unesterifed form of cholesterol that was elevated and that this effect occurred even in the presence of a vast elevation in hepatic cholesterol caused by dietary cholesterol. The rate-limiting enzyme CYP7A that converts cholesterol to bile acids was not altered by ACOL, and the cholesterol transporter ABCG5 tended to be increased by ACOL, at least in OVX rats. Therefore, impaired cholesterol excretion does not appear to explain the small ACOL-induced accumulation of liver cholesterol, which is more likely due to a larger receptor-mediated uptake of cholesterol from the circulation that would not be fully processed through increased esterification/storage or biliary excretion.

In a previous study, a robust hypocholesterolemic effect of ACOL treatment was observed both in the HDL- and non-HDL fractions in rats fed with a cholesterol-free diet, despite relatively low basal cholesterol levels [8]. Our previous observation that ACOL increases both liver SR-B1, which is involved in selective uptake of cholesterol from HDL [47-50], and the LDLr, without affecting HMG-CoA reductase or ACAT activity, strongly suggests that the SERM reduces cholesterolemia through favoring liver cholesterol uptake from all circulating lipoproteins in rats fed with a cholesterol-free diet. Dietary cholesterol down-regulates both the SR-B1 and LDLr [16,19], and it was therefore of interest to assess whether ACOL would remain able to lower cholesterolemia under such long- and short-term (refeeding) dietary challenge. The present findings clearly demonstrate that ACOL does strongly reduce cholesterolemia under these conditions. The SERM was equally effective in intact and OVX animals, in congruence with its very strong affinity for both estrogen receptors

α and β [51]. Because high levels of dietary cholesterol strongly reduce cholesterol transported by the HDL fraction, and because all of the hypocholesterolemic actions of ACOL under these conditions are exerted on the non-HDL fraction, further attention was given here to determinants of hepatic cholesterol synthesis/storage and the LDLr, the latter being largely responsible for hepatic uptake of non-HDL.

Acolbifene did not modulate key enzymes of cholesterol synthesis. The activity of the rate-limiting enzyme HMG-CoA reductase was not affected by the SERM, nor were mRNA levels of the SREBP proteins, which are master regulators of the cholesterol (mainly SREBP-2) and triglyceride (SREBP-1a and SREBP-1c) synthetic pathways [21]. Instead, ACOL treatment was associated with an increase in LDLr protein abundance, suggesting the involvement of hepatic lipoprotein uptake in its hypocholesterolemic action. In rats fed with a cholesterol-free diet [10], ACOL brought about a 2-fold elevation in liver LDLr protein that was independent of the nutritional status. That the ACOL-induced increase in LDLr was more marked in fasted than in fed rats here suggests that short-term cholesterol intake may transiently reduce the efficiency with which ACOL up-regulates the receptor. Of note is the fact that the SERM did not alter LDLr mRNA levels, indicating translational or posttranslational modulation. The LDLr is well known to be largely regulated at the level of gene expression [52]; however, ample evidence of posttranscriptional regulation exists for modulators such as estrogen, statins, and dietary cholesterol [53-57].

The ACOL-induced increase in hepatic LDLr content obviously does not exclude the possibility of other pathways being involved in the hypocholesterolemic action of the SERM. Interestingly, ACOL tended to increase liver expression of ABCG5 in OVX rats. The ABCG5/ABCG8 heterodimer constitutes a key cholesterol transporter in both the liver and intestine [58-61]. More recently, the Niemann-Pick C1 Like 1 protein was identified as a major inward intestinal cholesterol transporter [62]. Whether steroid hormones in general, and SERMs such as ACOL in particular, modulate these sterol transporters, in addition to hepatic lipoprotein receptors, constitutes an attractive possibility that needs further investigation.

In summary, the powerful cholesterol-lowering action of the SERM ACOL previously demonstrated in rats fed with cholesterol-free diets was shown here to be maintained postprandially in rats fed with a high-cholesterol diet and to be equally efficacious in the presence or absence of ovarian steroids. Assessment of major modulators of hepatic cholesterol metabolism suggests that the mechanisms of action of ACOL in cholesterol-fed rats are independent from the cholesterol synthetic pathway and may at least partly involve the translational or posttranslational up-regulation of the LDLr. Acolbifene therefore acts upon hepatic cholesterol metabolism through pathways that are entirely different from those of conventional hypocholesterolemic drugs. If ACOL retains such efficacy in humans, it may provide an attractive means of preventing, among others, the deleterious modifications of lipoprotein metabolism associated with menopause.

Acknowledgment

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to YD.

The authors gratefully acknowledge the expert technical assistance of Sébastien Poulin, Marie-Noëlle Cyr, and Céline Martel.

References

[1] Law MR, Wald NJ. Risk factor thresholds: their existence under scrutiny. BMJ 2002;324:1570-6.
[2] Leys D, Deplanque D, Mounier-Vehier C, et al. Stroke prevention: management of modifiable vascular risk factors. J Neurol 2002;249:507-17.
[3] Després J-P, Lemieux I, Tchernof A, et al. Fat distribution and metabolism. Diabetes Metab 2001;27:209-14.
[4] Toth MJ, Tchernof A, Sites CK, et al. Menopause-related changes in body fat distribution. Ann N Y Acad Sci 2000;904:502-6.
[5] Tchernof A, Poehlman ET, Després J-P. Body fat distribution, the menopause transition, and hormone replacement therapy. Diabetes Metab 2000;26:12-20.
[6] Luo S, Sourla A, Labrie C, et al. Combined effects of dehydroepiandrosterone and EM-800 on bone mass, serum lipids, and the development of dimethylbenz(A)anthracene-induced mammary carcinoma in the rat. Endocrinology 1997;138:4435-44.
[7] Picard F, Deshaies Y, Lalonde J, et al. Effects of the estrogen antagonist EM-652.HCl on energy balance and lipid metabolism in ovariectomized rats. Int J Obes Relat Metab Disord 2000;24:830-40.
[8] Lemieux C, Picard F, Labrie F, et al. The estrogen antagonist EM-652 and dehydroepiandrosterone prevent diet- and ovariectomy-induced obesity. Obes Res 2003;11:477-90.
[9] Labrie F, El-Alfy M, Berger L, et al. The combination of a novel selective estrogen receptor modulator with an estrogen protects the mammary gland and uterus in a rodent model: the future of postmenopausal women's health? Endocrinology 2003;144:4700-6.
[10] Lemieux C, Gélinas Y, Lalonde J, et al. The hypolipidemic action of the selective estrogen receptor modulator acolbifene is associated with decreased liver MTP and increased SR-B1 and LDL receptors. J Lipid Res 2005;46:1285-94.
[11] Lemieux C, Gélinas Y, Lalonde J, et al. The selective estrogen receptor modulator acolbifene reduces cholesterolemia independently of its anorectic action in control and cholesterol-fed rats. J Nutr 2005;135:2225-9.
[12] Kovanen PT, Brown MS, Goldstein JL. Increased binding of low density lipoprotein to liver membranes from rats treated with 17 alpha–ethinyl estradiol. J Biol Chem 1979;254:11367-73.
[13] Chao YS, Windler EE, Chen GC, et al. Hepatic catabolism of rat and human lipoproteins in rats treated with 17 alpha–ethinyl estradiol. J Biol Chem 1979;254:11360-6.
[14] Windler EE, Kovanen PT, Chao YS, et al. The estradiol-stimulated lipoprotein receptor of rat liver. A binding site that membrane mediates the uptake of rat lipoproteins containing apoproteins B and E. J Biol Chem 1980;255:10464-71.
[15] Erickson SK, Jaeckle S, Lear SR, et al. Regulation of hepatic cholesterol and lipoprotein metabolism in ethinyl estradiol–treated rats. J Lipid Res 1989;30:1763-71.
[16] Fluiter K, van der Westhuijzen DR, van Berkel TJ. In vivo regulation of scavenger receptor BI and the selective uptake of high density

lipoprotein cholesteryl esters in rat liver parenchymal and Kupffer cells. J Biol Chem 1998;273:8434-8.
[17] DeLamatre JG, Krause BR, Wong L, et al. A single high-cholesterol, high-fat meal preferentially increases low molecular weight apolipoprotein B concentration in rat plasma. J Lipid Res 1985;26:924-9.
[18] Fungwe TV, Cagen LM, Cook GA, et al. Dietary cholesterol stimulates hepatic biosynthesis of triglyceride and reduces oxidation of fatty acids in the rat. J Lipid Res 1993;34:933-41.
[19] Ness GC, Gertz KR. Hepatic HMG-CoA reductase expression and resistance to dietary cholesterol. Exp Biol Med (Maywood) 2004; 229:412-6.
[20] Horton JD, Shimomura I. Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. Curr Opin Lipidol 1999;10:143-50.
[21] Edwards PA, Tabor D, Kast HR, et al. Regulation of gene expression by SREBP and SCAP. Biochim Biophys Acta 2000;1529:103-13.
[22] Folch J, Lees M, Sloane G, Stanley H. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.
[23] Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
[24] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
[25] Shimomura I, Bashmakov Y, Ikemoto S, et al. Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. Proc Natl Acad Sci U S A 1999;96:13656-61.
[26] Richard D. Effects of ovarian hormones on energy balance and brown adipose tissue thermogenesis. Am J Physiol Regul Integr Comp Physiol 1986;250:R245-9.
[27] Wade GN, Gray JM, Bartness TJ. Gonadal influences on adiposity. Int J Obes Relat Metab Disord 1985;9(Suppl 1):83-92.
[28] Deshaies Y, Dagnault A, Lalonde J, et al. Interaction of corticosterone and gonadal steroids on lipid deposition in the female rat. Am J Physiol Endocrinol Metab 1997;273:E355-62.
[29] Dagnault A, Deshaies Y, Richard D. Involvement of type I corticosteroid receptor in the effects of ovariectomy on energy balance. Am J Physiol Regul Integr Comp Physiol 1996;270:R199-R206.
[30] Asarian L, Geary N. Cyclic estradiol treatment phasically potentiates endogenous cholecystokinin's satiating action in ovariectomized rats. Peptides 1999;20:445-50.
[31] Geary N. Estradiol, CCK and satiation. Peptides 2001;22:1251-63.
[32] Zak Z, Lagrost L, Gautier T, et al. Expression of simian CETP in normolipidemic Fisher rats has a profound effect on large sized apoE-containing HDL. J Lipid Res 2002;43:2164-71.
[33] Gorinstein S, Bartnikowska E, Kulasek G, et al. Dietary persimmon improves lipid metabolism in rats fed diets containing cholesterol. J Nutr 1998;128:2023-7.
[34] Abbey M, Triantafilidis C, Topping DL. Dietary non-starch polysaccharides interact with cholesterol and fish oil in their effects on plasma lipids and hepatic lipoprotein receptor activity in rats. J Nutr 1993;123:900-8.
[35] Krause BR, Newton RS. Apolipoprotein changes associated with the plasma lipid-regulating activity of gemfibrozil in cholesterol-fed rats. J Lipid Res 1985;26:940-9.
[36] Cole TG, Kuisk I, Patsch W, et al. Effects of high cholesterol diets on rat plasma lipoproteins and lipoprotein-cell interactions. J Lipid Res 1984;25:593-603.
[37] Fungwe TV, Cagen L, Wilcox HG, et al. Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol. J Lipid Res 1992;33:179-91.
[38] Lyons PM, Truswell AS, Mira M, et al. Reduction of food intake in the ovulatory phase of the menstrual cycle. Am J Clin Nutr 1989; 49:1164-8.
[39] Mannucci E, Ognibene A, Becorpi A, et al. Relationship between leptin and oestrogens in healthy women. Eur J Endocrinol 1998; 139:198-201.
[40] Bertolotti M, Spady DK. Effect of hypocholesterolemic doses of 17a–ethinyl estradiol on cholesterol balance in liver and extrahepatic tissues. J Lipid Res 1996;37:1812-22.
[41] Parini P, Angelin B, Stavreus-Evers A, et al. Biphasic effects of the natural estrogen 17b-estradiol on hepatic cholesterol metabolism in intact female rats. Arterioscler Thromb Vasc Biol 2000;20: 1817-23.
[42] El-Swefy SE, Asker ME, Ali SI, et al. A novel concept to preserve the beneficial effects of hormone replacement therapy in bilaterally female ovariectomized rats: role of lovastatin therapy. Pharmacol Res 2002;45:167-73.
[43] Mauriège P, Martel C, Langin D, et al. Chronic effects of dehydroepiandrosterone on rat adipose tissue metabolism. Metabolism 2003;52:264-72.
[44] Nuttall ME, Bradbeer JN, Stroup GB, et al. Idoxifene: a novel selective estrogen receptor modulator prevents bone loss and lowers cholesterol levels in ovariectomized rats and decreases uterine weight in intact rats. Endocrinology 1998;139:5224-34.
[45] Uesugi T, Toda T, Tsuji K, et al. Comparative study on reduction of bone loss and lipid metabolism abnormality in ovariectomized rats by soy isoflavones, daidzin, genistin, and glycitin. Biol Pharm Bull 2001; 24:368-72.
[46] Cazita PM, Berti JA, Aoki C, et al. Cholesteryl ester transfer protein expression attenuates atherosclerosis in ovariectomized mice. J Lipid Res 2003;44:33-40.
[47] Acton S, Rigotti A, Landschulz KT, et al. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 1996; 271:518-20.
[48] Ji Y, Jian B, Wang N, et al. Scavenger receptor BI promotes high density lipoprotein–mediated cellular cholesterol efflux. J Biol Chem 1997;272:20982-5.
[49] Stangl H, Hyatt M, Hobbs HH. Transport of lipids from high and low density lipoproteins via scavenger receptor-BI. J Biol Chem 1999; 274:32692-8.
[50] Gu X, Kozarsky K, Krieger M. Scavenger receptor class B, type I-mediated [3H]cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. J Biol Chem 2000;275:29993-30001.
[51] Labrie F, Labrie C, Bélanger A, et al. EM-652 (SCH 57068), a third generation SERM acting as pure antiestrogen in the mammary gland and endometrium. J Steroid Biochem Mol Biol 1999; 69:51-84.
[52] Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425-30.
[53] Lopez D, McLean MP. Sterol regulatory element-binding protein–1a binds to *cis* elements in the promoter of the rat high density lipoprotein receptor SR-BI gene. Endocrinology 1999;140:5669-81.
[54] Ness GC, Zhao Z, Lopez D. Inhibitors of cholesterol biosynthesis increase hepatic low-density lipoprotein receptor protein degradation. Arch Biochem Biophys 1996;325:242-8.
[55] Roach PD, Balasubramaniam S, Hirata F, et al. The low-density lipoprotein receptor and cholesterol synthesis are affected differently by dietary cholesterol in the rat. Biochim Biophys Acta 1993; 1170:165-72.
[56] Landschulz KT, Pathak RK, Rigotti A, et al. Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. J Clin Invest 1996;98:984-95.
[57] Srivastava RA, Baumann D, Schonfeld G. In vivo regulation of low-density lipoprotein receptors by estrogen differs at the post-transcriptional level in rat and mouse. Eur J Biochem 1993; 216:527-38.
[58] Berge KE, Tian H, Graf GA, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science 2000;290:1771-5.
[59] Graf GA, Yu L, Li WP, et al. ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. J Biol Chem 2003;278:48275-82.

[60] Wilund KR, Yu L, Xu F, et al. High-level expression of ABCG5 and ABCG8 attenuates diet-induced hypercholesterolemia and atherosclerosis in Ldlr−/− mice. J Lipid Res 2004;45: 1429-36.

[61] Yu L, Li-Hawkins J, Hammer RE, et al. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J Clin Invest 2002; 110:671-80.

[62] Davis HR, Zhu LJ, Hoos LM, et al. Niemann-pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. J Biol Chem 2004;279:33586-92.