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Research Paper

Acute P-407 Administration to Mice Causes Hypercholesterolemia by Inducing Cholesterolgenesis and Down-Regulating Low-Density Lipoprotein Receptor Expression

Carlos Leon,[1] Kishor M. Wasan,[1] Kristina Sachs-Barrable,[1] and Thomas P. Johnston[2,3]

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Purpose. Poloxamer 407 (P-407) is a chemical that induces a dose-controlled dyslipidemia in mice. Our aim was to determine the acute effects of P-407 treatment on the mechanisms that influence hepatic cholesterol homeostasis.
Methods. We measured lipid levels in plasma and liver samples from control and P-407-treated mice (24 h post-i.p. injection of 0.5 g kg^{-1} of P-407 or saline for the control mice). We measured acyl-coenzyme A:cholesterol acyltransferase (ACAT) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activities in liver microsomes. The protein expression of ACAT2, scavenger receptor class B, type I (SR-BI), ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G8 (ABCG8), low-density lipoprotein receptor (LDLr), and actin was measured by immunoblot.
Results. We found an increase in plasma cholesterol and triglyceride levels as well as increased hepatic cholesteryl esters (CE) in P-407-treated mice. The hepatic ACAT microsomal activity and ACAT2 protein expression were not altered by P-407. The protein expression of the LDLr was decreased in the livers of P-407-treated mice. This decrease was specific, because the expression of the SR-BI was unchanged. The P-407-induced hypercholesterolemia was accounted for by increased activity and protein expression of HMG-CoA reductase. ATP-binding cassette transporters A1 and G8 protein expression were not significantly different in P-407-treated mice compared to controls.
Conclusions. The increased hepatic CE levels, following P-407 treatment, was neither related to an up-regulation of ACAT2 nor enhanced SR-BI expression. Hypercholesterolemia was associated with an up-regulation of both the protein expression and activity of HMG-CoA reductase and decreased LDLr expression.

KEY WORDS: cellular cholesterol efflux; P-407-induced dyslipidemia; reverse cholesterol transport.

INTRODUCTION

While cholesterol is an essential component of mammalian cell membranes, excess cellular cholesterol is toxic and contributes to several diseases, most notably atherosclerosis. The development of atherosclerosis has been studied in a variety of animal models that simulate the human condition (1,2). Recently, mouse models of atherogenesis have gained popularity because precise genetic alterations are possible that assist in identifying key factors involved with atherosclerosis. However, these knockout mouse models, such as the LDL receptor and ApoE knockout models, have limitations. Although these models help to identify various factors involved in atherosclerotic lesion development, they are a drastic departure from normal physiology and the human condition. Previously, we have reported on a nongenetically altered, nondiet-induced mouse model of atherosclerosis (3). Our animal model involves the parenteral administration of a compound called poloxamer 407 (P-407), a hydrophilic triblock copolymer comprising polyoxyethylene and polyoxypropylene units, to 5- to 6-week-old male and female C57BL/6 mice. A dose-dependent dyslipidemia is observed as early as 1 h following P-407 administration and lasts approximately 4–5 days following a single dose. The changes observed in plasma and tissue lipid concentrations, as well as modulations in the activity and expression of key enzymes involved with lipid metabolism, are very similar in P-407-treated mice and rats. If chronic administration of P-407 to mice is maintained

[1] Division of Pharmaceutics and Biopharmaceutics, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada.
[2] Division of Pharmaceutical Sciences, Rm. 211A, School of Pharmacy, University of Missouri–Kansas City, 5005 Rockhill Road, Kansas, Missouri 64110-2499, USA.
[3] To whom correspondence should be addressed. (e-mail: johnstont@umkc.edu

ABBREVIATIONS: HDL, high-density lipoprotein; CE, cholesteryl ester; ACAT, acyl-coenzyme A:cholesterol acyltransferase; LCAT, lecithin cholesterol acyltransferase; SR-BI, scavenger receptor class B, type I; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ABCA1, ATP-binding cassette transporter A1; ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; CHD, coronary heart disease; LXR, liver X receptor; P-407, poloxamer 407; LDLr, low-density lipoprotein receptor.

for 16 weeks, aortic atherosclerotic lesions are formed that are comparable in size and number to those observed when using classic high-fat, diet-induced mouse models of atherogenesis (3). The P-407 model of hyperlipidemia has been validated by other research groups in mice (4) and in rabbit (5). In particular, Millar *et al.* (4) used the mouse P-407 model to measure triglyceride production and compared it favorably with the current method, which uses Triton WR-1339. Brocks *et al.* (6) used the P-407 murine model to analyze the effect of dyslipidemia in Cyclosporine A lipoprotein distribution.

Understanding of cellular cholesterol homeostasis and its regulation has unfolded over several decades (7). As it pertains to coronary heart disease (CHD) secondary to arteriosclerosis, the focus of research has now shifted to cellular cholesterol efflux and reverse cholesterol transport, whereby cholesterol is transported from peripheral cells to the liver for elimination into bile. Oxysterol derivatives of cholesterol that accumulate in cells when intracellular cholesterol concentrations are elevated, are ligands for the liver X receptor (LXR) nuclear receptors, which stimulate the expression of ATP-binding cassette transporters (ABCA1, ABCG1, ABCG5/ABCG8) and other genes involved in reverse cholesterol transport (1). Several LXR agonists are now being evaluated for their potential to increase the gene expression of ABCA1, which, in turn, increases the efflux of excess cellular cholesterol to lipid-poor HDL for its eventual return to the liver.

The primary purpose of this study was to investigate the putative mechanisms that might explain our previous finding of increased plasma cholesterol levels and tissue CE concentration in P-407-treated rodents. Thus we assessed the activity and/or protein expression of scavenger receptor class B, type I (SR-BI), LDLr, acyl-coenzyme A:cholesterol acyltransferase (ACAT), HMG-CoA reductase, ABCG8, and ABCA1 following P-407 administration to mice.

METHODS

Materials

Poloxamer 407 (P-407) was a gift from the BASF Corporation (Parsippany, NJ, USA) and used as received. Tritiated mevalonolactone (30 Ci $mmol^{-1}$), [^{14}C]3-hydroxy-3-methylglutaryl coenzyme A (57 mCi $mmol^{-1}$), [^{14}C]oleoyl-CoA (55 mCi $mmol^{-1}$), and ECL immunodetection reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). Solvents for thin-layer chromatography (TLC) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Nitrocellulose, protein assay kits, and other electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA). NADPH, HCl, KCl, aprotinin, dithiothreitol, ethylenediamine tetraacetic acid, and sucrose were purchased from Sigma (St. Louis, MO, USA) and used as received. 3-[Decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenethyl] propanamide or Sandoz 58-035 was obtained from Sigma-Aldrich (St. Louis, MO, USA). RNeasy Mini kits (Pdt. No. 74104) were obtained from Qiagen (Valencia, CA, USA). Male C57BL/6 (B6) mice (22–23 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Assay reagents for the determination of total and free cholesterol, as well as triglycerides, were obtained from Wako Diagnostics (Richmond, VA, USA).

P-407 Administration

Mice were maintained for 1 week in a temperature-controlled room (22°C) prior to experimentation. All animals were provided standard rodent chow and water *ad libitum*. The procedures for P-407 administration and subsequent blood and tissue collection were in accordance with the provisions of the institution's guide for the care and use of laboratory animals, and the treatment protocol was approved by the Animal Care and Use Committee (IACUC) at the University of Missouri–Kansas City. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

On the day of the experiment, each mouse was administered a 0.5 g kg^{-1} dose of P-407 (0.5 mL) by intraperitoneal (i.p.) injection. Mice ($n = 4$) were fasted over the 24 h following P-407 administration to avoid contribution of postprandial lipids to the plasma lipid levels. Previously, we have reported that the plasma total cholesterol concentration reaches a maximum (C_{max}) 24 h following an i.p. injection of P-407 (8). Thus, liver, small intestine, and plasma samples were all harvested 24 h postdosing. The control mice ($n = 4$) were each administered 0.5 mL of sterile normal saline by i.p. injection and similarly treated.

Tissue Collection

On the day of sacrifice (24 h after receiving either a dose of P-407 or normal saline), blood samples were obtained by periorbital sampling (0.8 mL $sample^{-1}$) under ether anesthesia. All blood samples were collected into either heparinized (100 units mL^{-1}) 1.5-mL polypropylene tubes and immediately placed on ice. After blood collection, the animal was immediately sacrificed by cervical dislocation while under ether anesthesia. The blood samples were centrifuged at 10,000 g for 10 min at 4°C; plasma was harvested and plasma samples were frozen at −80° until the time of analysis.

Small samples (~250–500 mg) of liver and small intestine were immediately obtained *postmortem* from all animals. Excised tissue samples were placed in individual tared cryogenic polypropylene vials and reweighed to obtain the exact wet weight of the tissue specimen. All samples were flash-frozen on dry ice and then stored at −80°C until the time of analysis.

Lipid Analysis

To confirm that the dose of P-407 injected into the peritoneal cavity was absorbed and elicited its hyperlipidemic effect, frozen plasma samples were thawed and vortexed, and 20-μL aliquots were analyzed for total cholesterol by using a standard enzymatic, colorimetric assay kit. The assay is based on the technique described by Allain *et al.* (9), in which cholesterol is oxidized in a reaction catalyzed by cholesterol

oxidase. A red-colored quinone pigment is ultimately generated and its absorbance determined at 505 nm. Plasma samples were assayed to assure that the concentration of total cholesterol was within 1 standard deviation of the previously reported mean value of C_{max}. Triglyceride concentrations were measured by the method of Bucolo and David (10) with a standard enzymatic, colorimetric-based assay. All assays for total cholesterol and triglyceride were performed in duplicate.

For analysis of total and free cholesterol in liver specimens, samples were thawed and subsequently homogenized in a 3:1 ethanol/ether mixture as described by Entenman (11). The samples were then evaporated to dryness at 90°C. Residues were solubilized with 3 mL 2-propanol and assayed as described above for total cholesterol in plasma. The cholesterol standard provided with the kit had a concentration of 100 mg mL^{-1} and was also dissolved in 2-propanol. Pilot studies demonstrated good linearity for cholesterol quantification in 2-propanol and recoveries near 100% using the cholesterol assay kits. The CE content of the liver was simply calculated as the difference between the total and free cholesterol concentrations. The concentrations of total cholesterol, free cholesterol, and CE in the liver were expressed in units of mg g^{-1} of tissue.

ACAT Activity Measurements

For ACAT activity determinations, the assay described by Rudel *et al.* (12–14) was used. Using standard techniques (12), hepatic microsomes were prepared from freshly harvested livers obtained 24 h after treatment with either P-407 (0.5 g kg^{-1}) ($n = 4$) or saline ($n = 4$). The concentration of microsomal protein resuspended in ACAT buffer (0.1 M Tris–HCl, 0.25 M sucrose, and 1 mM EDTA, pH 7.5) was measured by the method of Lowry *et al.* (15), and 100-μL aliquots at a concentration of approximately 0.5–1.0 mg mL^{-1} were stored at −80°C until the time of assay. A curve for ACAT activity as a function of different amounts of microsomal protein was generated (25–100 μg microsomal protein). The specificity of the assay was corroborated by using the ACAT inhibitor, Sandoz 58-035, which completely abrogated the radioactive incorporation into the cholesteryl ester band (data not shown). Microsomes were isolated at least twice from each animal and then assayed by duplicate or triplicate to obtain representative results.

Microsomes were thawed and an aliquot containing 50 μg protein was mixed with 335 μg BSA, and the final volume was brought to 200 μL by using ACAT buffer. The sample was equilibrated in a 37°C water bath for 5 min, then 10 nmol [^{14}C]oleoyl-CoA was added to the tube and the mixture was incubated for 3 min in a shaking water bath. The reaction was stopped with the addition of 2 mL of a 2:1 (v:v) chloroform–methanol solution. Cholesteryl oleate and radioactive cholesterol were added as a carrier and to calculate lipid recovery, respectively. Next, 1 mL of a sodium chloride solution (0.9%) was added, and the samples were vortexed for 1 min and incubated for 1 h at 37°C. Afterwards, they were allowed to sit overnight at 4°C. The lower layer containing the product was then removed and evaporated to dryness under nitrogen. The residue was resuspended in 50 μL chloroform and then applied to a TLC plate with subsequent separation in hexane–ethyl acetate (9:1). Lipids were visualized with iodine vapors and the portion of the TLC plate containing the CE was scraped and suspended in scintillation fluid, and the radioactivity determined. The data was expressed as specific activity (pmol min^{-1} mg^{-1} of microsomal protein).

HMG-CoA Reductase Activity Measurements

This experiment was conducted to quantify the enzymatic activity of microsomal HMG-CoA reductase from livers isolated 24 h after the administration of P-407 or saline to mice. The activity of HMG-CoA reductase in hepatic microsomal homogenates was assayed by the method of Shapiro *et al.* (16). Microsomes, 5–100 μl (50 μg protein), which were gently resuspended by homogenization, were mixed at 4°C with homogenization buffer (27 μl; 30 mM EDTA, 250 mM NaCl, 1.0 mM DTT, 50 mM potassium phosphate pH 7.4) and water and brought to a final volume of 125 μL. After a 5-min preincubation at 37°C, 50 μL of the cofactor–substrate solution containing the radioactive substrate was added [4.5 μmol glucose 6-phosphate, 0.3 IU glucose 6-phosphate dehydrogenase, 450 nmol $NADP^+$, 50 nmol DL-hydroxymethyl [3^{-14}C]glutaryl CoA (specific activity 57 mCi $mmol^{-1}$)].

After 15 min at 37°C, incubations were terminated by adding 25 μL of 12 N HCl. Tritiated mevalonic acid was added as a recovery control. Samples were incubated at 37°C for at least 30 min to allow mevalonic acid to lactonize, and then centrifuged for 1 min to sediment the denatured protein. Portions (generally 50 of 200 μL) of the protein-free supernatant solution were then applied to silica gel TLC sheets (7.5 μL at a time). The chromatograph was developed in benzene-acetone (1:1, v/v) and then air-dried. The region R_f = 0.6–0.9 was removed and placed into two vials, each containing 10 mL scintillation fluid, and counted for both ^{14}C and ^{3}H. Raw ^{14}C data were corrected for recovery by using the data for the ^{3}H mevalonic acid internal control. Corrected recoveries ranged between 90 and 99%. Enzyme activities were expressed as pmoles of mevalonic acid synthesized per minute per milligram protein (pmol min^{-1} mg^{-1}).

ACAT2, HMG-CoA Reductase, SR-BI, Actin, ABCG8, and ABCA1 Protein Expression Assays

ACAT2

ACAT2 protein was determined in liver microsomes following the procedure described by Lee *et al.* (12), by using a rabbit polyclonal antibody kindly provided by Dr. L. Rudel (Wake Forest University, Winston-Salem, NC).

HMG-CoA reductase

The protein expression of HMG-CoA reductase was assessed in liver extracts. Mice livers were solubilized in RIPA buffer containing PMSF and other protease inhibitors and then centrifuged at 12,000 *g* for 15 min at 4°C. The supernatants were collected and stored at −70°C until analysis by SDS-PAGE and Western blotting. Briefly, 20 μg

of each extract was separated on a 7% acrylamide gel and transferred to a nitrocellulose membrane. After blocking in TBS 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder, the membranes were incubated with a 1:1,000 dilution in blocking buffer of a rabbit polyclonal antibody, anti-HMG-CoA reductase (Upstate Biotechnology), for 2 h at room temperature. The membranes were washed in TBS-T and then incubated with a 1:3,000 dilution of a goat antirabbit HRP-conjugated secondary antibody (Bio-Rad) in TBS-T for 1 h. After the washings, the membranes were incubated with enhanced chemiluminescence (ECL) and processed via autoradiography.

Actin

The differences in protein loading between lanes were corrected by actin immunoblotting. Briefly, the membranes were blocked in 3% BSA in TBS-T for 1 h. After this period, the membranes were incubated with a 1:5,000 dilution in blocking buffer of a goat polyclonal antibody antiactin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Following the washings, the membranes were incubated (1:4,000 dilution) with a bovine antigoat HRP-conjugated secondary antibody (Santa Cruz Biotechnology) in TBS-T for 1 h. After subsequent washes, the bands were detected as described above.

SR-BI

The goat polyclonal antibody to the SR-BI (Novus Biologicals, Littleton, CO, USA), which recognizes a sequence between residues 1 and 100 of murine SR-BI (100% homologous to rat and human), was diluted 1:1,000 in blocking buffer (3% BSA, 0.1% Tween 20 and 1× TBS) plus azide. The antibody and membrane were incubated overnight in rotation at 4°C. The secondary antibody incubation and development were performed similar to the procedure described for actin.

ABCA1, ABCG8, and LDLr

The protocol used to detect these proteins in liver extracts was basically the same as that described for HMG-CoA reductase. The rabbit polyclonal antibodies, anti-ABCA1, anti-ABCG8 (both from Novus Biologicals), and anti-LDLr (Research Diagnostics, Flanders, NJ, USA) were diluted 1:1,000 in TBS-T 5% milk. The membrane was incubated with primary antibody for 2 h, washed, and incubated for 1 h with goat antirabbit HRPO diluted 1:3,000 in blocking solution.

Statistical Analysis

Mean values of ACAT and HMG-CoA reductase activities and normalized protein expression, as well as plasma and liver lipid concentrations, for P-407- and saline-treated mice were compared for statistical differences by using the ANOVA test (INSTAT; GraphPad, San Diego, CA, USA). This program employs a Tukey *post-hoc* test. A difference in mean values was considered significant if $p < 0.05$. All data are expressed as mean ± SD.

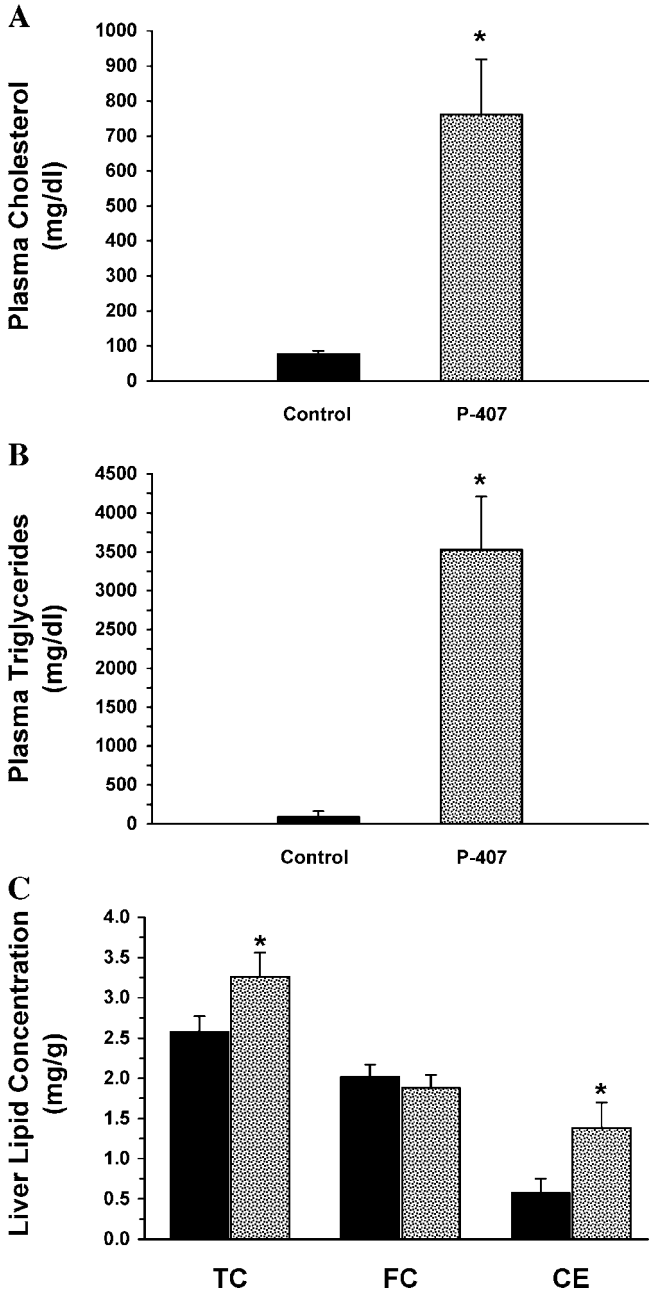

Fig. 1. (A) Plasma cholesterol concentrations in control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice 24 h after administration of P-407. Bars represent the mean value ± SD. Asterisk (*) indicates a significant ($p < 0.01$) increase compared to control. (B) Plasma triglyceride concentrations in control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice 24 h after administration of P-407. Bars represent the mean value ± SD. Asterisk (*) indicates a significant ($p < 0.01$) increase compared to control. (C) Liver total cholesterol, free cholesterol, and CE concentrations in control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice 24 h after administration of P-407. Bars represent mean value ± SD. Asterisk (*) indicates a significant ($p < 0.01$) increase compared to control.

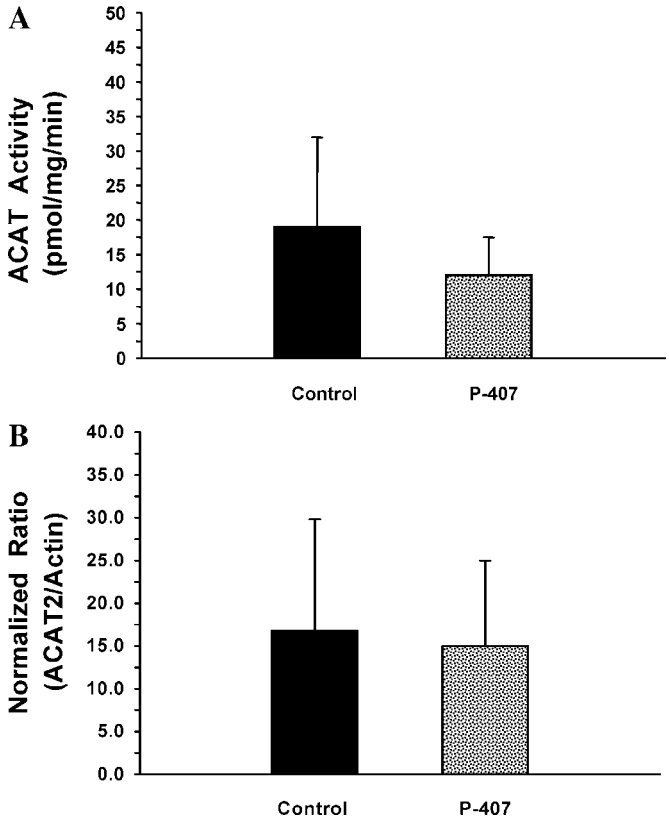

Fig. 2. (A) ACAT2 activity in hepatic microsomes obtained from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed by 3–5 replicates. Bars represent mean value ± SD. No significant difference was observed. (B) ACAT2 protein expression in hepatic microsomes from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed by duplicate. Bars represent mean value ± SD. No significant difference was observed.

RESULTS

Cholesterol and Triglyceride Concentrations in Plasma and Liver

As can be noted in Fig. 1A and B, the plasma concentrations of total cholesterol and triglycerides, respectively, 24 h after P-407 administration were significantly greater for P-407-treated mice compared to controls. Figure 1C shows that total cholesterol is significantly increased in P-407-treated animals compared to controls. However, this difference is attributable to an increase in hepatic CE levels, because the liver free cholesterol content was not significantly different for P-407-treated mice, relative to controls, at 24 h postinjection.

ACAT Activity and Expression Measurements

Microsomes from untreated mice livers were isolated and characterized by using immunoblotting techniques to detect microsomal markers such as HMG-CoA reductase and Cyp2C11 (data not shown). The microsomes were used to determine ACAT activity as described earlier.

As can be noted in Fig. 2A, the enzymatic activity of ACAT assayed in hepatic microsomes of P-407-treated mice is similar to the corresponding measurement determined for controls. Likewise, P-407 administration to mice did not result in a significant difference in the protein expression of ACAT2, the main liver microsomal isoform, relative to controls (Fig. 2B).

SR-BI, LDLr, ABCG8, and ABCA1 Protein Expression Following P-407 Administration

Using mice liver extracts, Fig. 3A shows the immunoblot and normalized ratio of SR-BI to actin determined for both P-407-treated and control mice. As can be noted in Fig. 3A, no significant difference was observed. However, when the LDLr protein expression was assessed by immunoblotting, the P-407-treated liver extracts showed decreased LDLr levels. Figure 4 shows the immunoblot and normalized ratio of ABCA1 to actin determined for P-407-treated and control mice livers. No significant difference was observed that may account for enhanced plasma cholesterol levels in P-407-treated mice. Similarly, we assessed ABCG8 protein expression levels for P-407-treated and control mice liver extracts

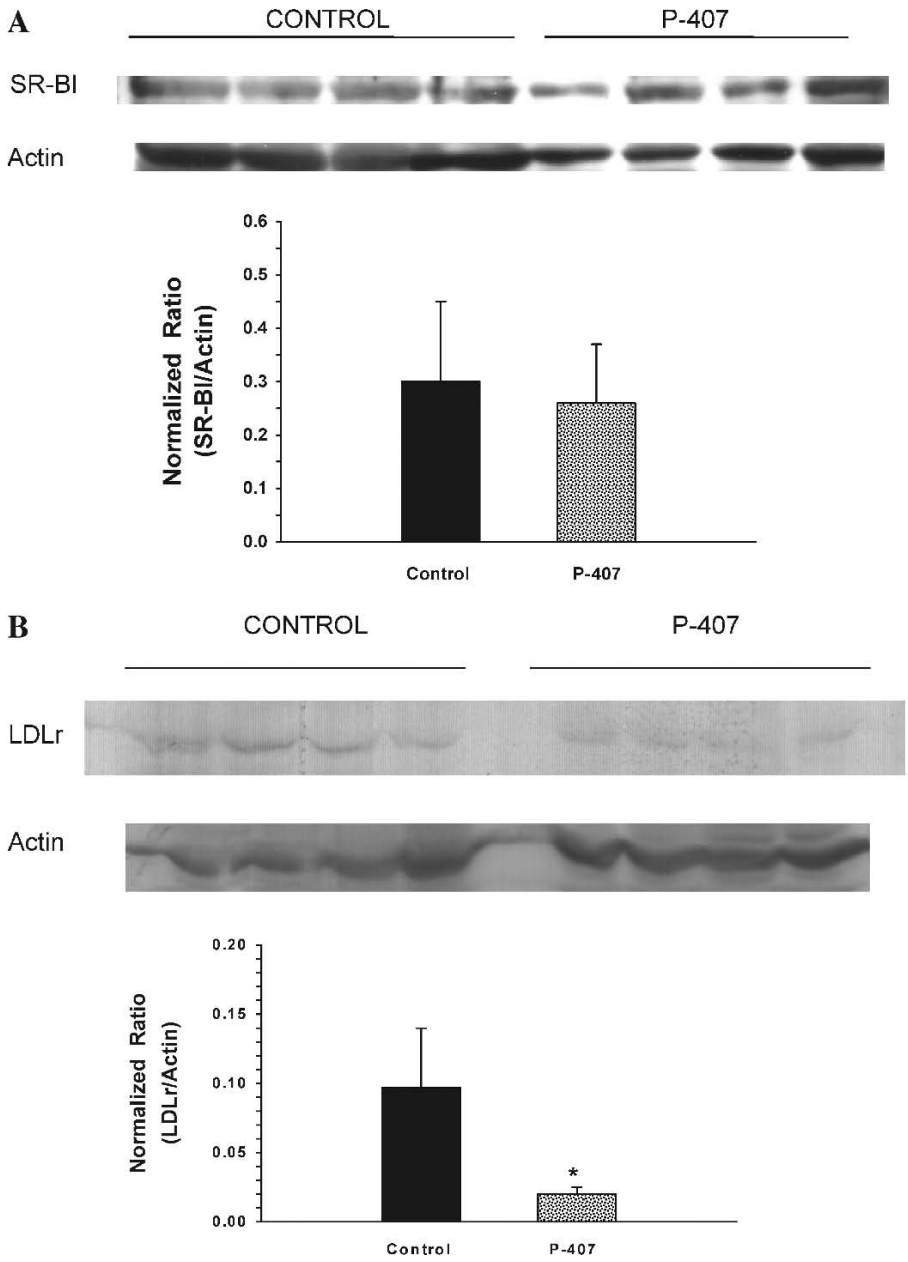

Fig. 3. (A) SR-BI protein expression in liver extracts obtained from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed by duplicate. Bars represent mean value ± SD. No significant difference was observed. (B) LDL receptor protein expression in liver extracts obtained from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed by duplicate. Bars represent mean value ± SD. Asterisk (*) indicates a significant ($p < 0.01$) decrease compared to control.

and we did not observe a significant difference between the two groups (data not shown).

HMG-CoA Reductase Activity and Protein Expression in Mice Liver Extracts Following P-407 Administration

Figure 5 shows a significant ($p < 0.01$) increase in the hepatic microsomal activity of HMG-CoA reductase 24 h after the administration of P-407 to mice. Similar to HMG-CoA reductase activity, the protein expression of HMG-CoA reductase was significantly ($p < 0.01$) greater than the corresponding mean value determined for controls at the same point (Fig. 6).

DISCUSSION

The present investigation was undertaken to determine if the activity and/or expression of key enzymes involved with lipid metabolism were modulated after the administration of P-407 to mice. Our aim was to determine the acute effects of P-407 treatment on the mechanisms influencing hepatic cholesterol homeostasis. We found an increase in

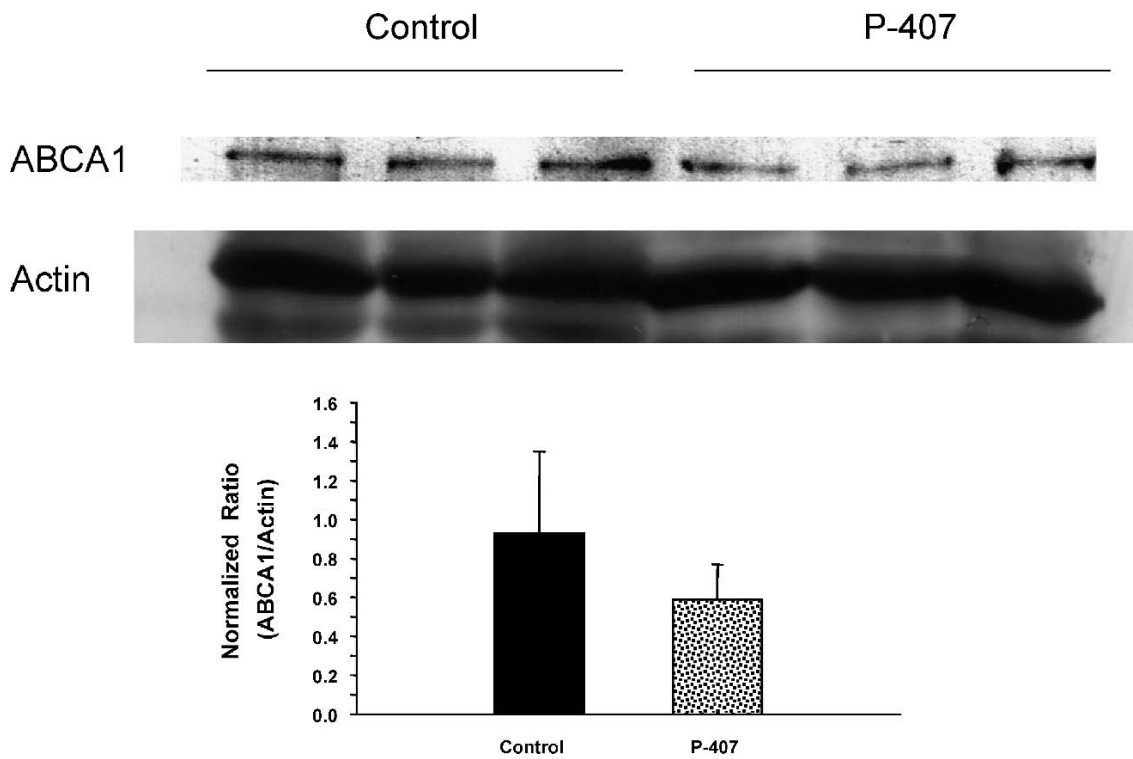

Fig. 4. ABCA1 protein expression in liver extracts obtained from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed by duplicate. Bars represent mean value ± SD. No significant difference was observed.

plasma cholesterol and triglyceride levels as well as increased hepatic cholesteryl esters (CE) in P-407-treated mice. The latter was similar to the elevated levels of CE we observed in the liver and eight peripheral (extrahepatic) tissues of P-407-treated rats (17). In that study, we also demonstrated that the concentration of cholesterol was significantly increased in the same tissues. Our working hypothesis in the present investigation was that the observed increase in the concentration of cholesterol was due to a P-407-mediated reduction in the amount of cholesterol effluxed from the cell and/or an increase in cellular cholesterol synthesis induced by P-407. The first part of our working hypothesis was based, in part, on our previous findings, which demonstrated a significant reduction in both apoA1-mediated cholesterol efflux for macrophages cultured with P-407. Regardless of whether increased cellular cholesterol was caused by impaired cholesterol efflux and/or increased cholesterol synthesis, our goal was to attempt to identify putative mechanisms responsible for the increased CE concentrations we detected in the liver and peripheral tissues of P-407-treated rodents.

Interestingly, although the administration of P-407 to mice results in increased levels of CE in the liver, the biological activity and expression of ACAT seems to be unaffected. Intuitively, one might expect that the activity and/or expression of ACAT would be up-regulated to compensate for increased cellular cholesterol levels arising from one or more of the following mechanisms: (a) increased cholesterol synthesis induced by P-407; (b) decreased cholesterol efflux due to a P-407-mediated reduction in the expression of ABCA1 and/or ABCG8; or (c) redistribution of excess plasma cholesterol (i.e., P-407-induced hypercholesterolemia) to the cells for storage. Of particular note, Wasan *et al.* (18) demonstrated that the biological activity of another acyltransferase—specifically, lecithin cholesterol acyltransferase (LCAT)—was increased 4- to 5-fold in the plasma of P-407-treated rats relative to controls. LCAT converts cholesterol to CE on the surface of high-density lipoproteins by catalyzing the transfer of fatty acids from the *sn-2* position of lecithin to the free hydroxyl group of

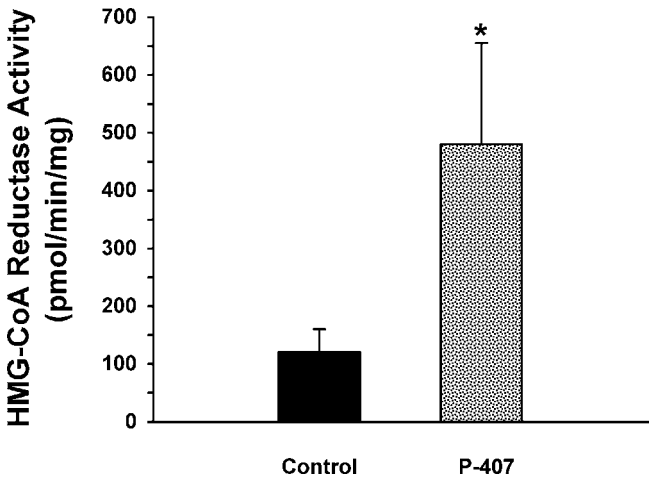

Fig. 5. HMG-CoA reductase activity in hepatic microsomes obtained from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed 3–5 times. Bars represent the mean value ± SD. Asterisk (*) indicates a significant ($p < 0.01$) increase compared to control.

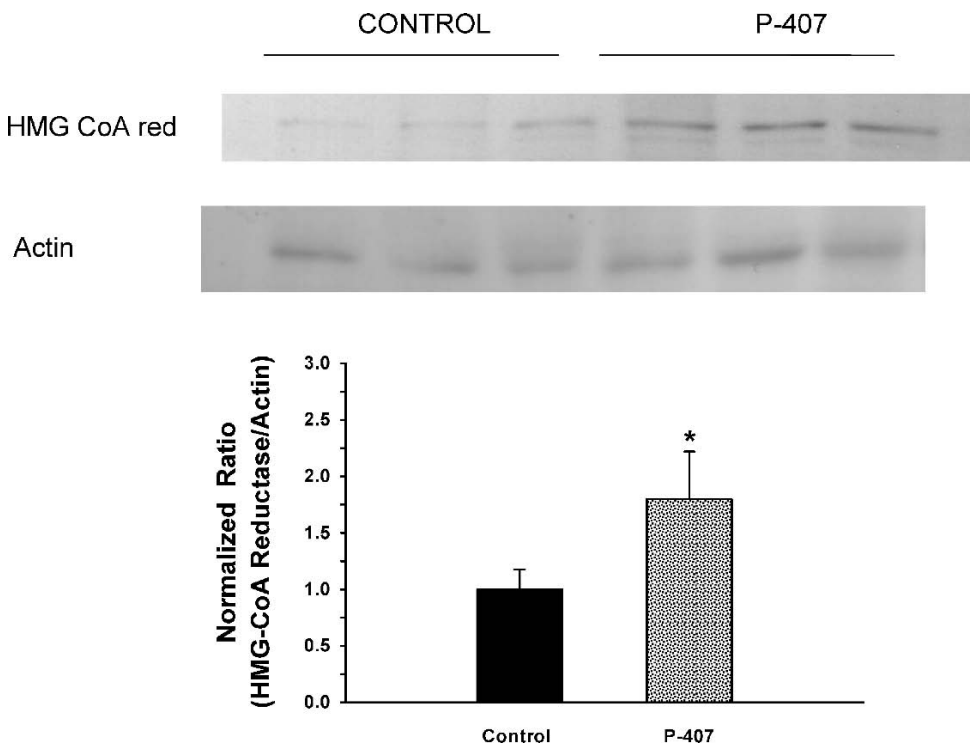

Fig. 6. HMG-CoA reductase protein expression in hepatic microsomes obtained from control (solid bar) and P-407-treated (0.5 g/kg) (stippled bar) mice. Each individual sample was analyzed by duplicate. Bars represent the mean value ± SD. Asterisk (*) indicates a significant ($p < 0.05$) increase compared to control.

cholesterol (19,20). As an aside, P-407 has also been shown to be an effective, broad-based lipase inhibitor (18,21,22) and, as such, exhibits no specificity for inhibiting one lipase over another. Therefore, it is interesting that P-407 administration to mice increases, either directly or indirectly, the biological activity of plasma LCAT, but does not alter either the activity, protein expression, or mRNA levels of ACAT.

Increased tissue CE concentrations in P-407-treated animals, especially as it relates to the liver, may be attributable to enhanced selective uptake of HDL-associated CE by SR-BI (23). We assessed the expression of SR-BI in liver extracts but we did not find a change in SR-BI protein expression associated with the P-407 treatment. However, we cannot discount the possibility that there was a change in influx in a scenario where the SR-BI transporters were not saturated.

The protein expression of the LDL receptor was markedly decreased in liver extracts from P-407-treated mice. Down-regulation of the LDL receptor coupled with a high cholesterol diet has been associated with the development of atherosclerosis (2).

As a potential mechanism to account for increased plasma cholesterol levels in P-407-treated animals, we tested the hepatic ABCA1 protein expression. This was based on our previous findings, which demonstrated a significant reduction in both apoA1-mediated cholesterol efflux and the gene expression of ABCA1 for macrophages cultured with P-407 (17). However, in the same study (17), ABCA1 protein expression in liver extracts from control and P-407-treated rats was analyzed and was not found to be significantly different. In the present work, we found no difference in hepatic ABCA1 protein expression between treatments, which is congruent with the study on rats. We also checked the protein expression of ABCG8, because it has been implicated, along with ABCG5, in the regulation of biliary cholesterol secretion (24). We did not find any difference in expression in control and P-407-treated mice hepatocytes, suggesting that ABCG8, and possibly ABCG5, are not likely to contribute to the elimination of cholesterol into bile, and may be one possible explanation, in conjunction with increased cholesterol biosynthesis, for the high levels of plasma cholesterol that we measured in the P-407-treated animals. The high plasma triglyceride levels can be explained by an inhibition of lipoprotein lipase the enzyme responsible for triglyceride degradation, as we have previously demonstrated in this model (22). Hepatic triglyceride levels were not measured in our study, but in a similar acute P-407 mouse model Millar *et al.* (4) do not report a significant change in triglyceride levels from untreated and P-407-treated mice livers.

In contrast to ACAT, the enzymatic activity, as well as the protein expression of HMG-CoA reductase, was increased after the administration of P-407 to mice. However, there seems to be a strong temporal relationship to both. For example, after 200 days of P-407 administration to mice (0.5 g kg^{-1} every 3 days by i.p. injection), we have previously shown that the activity of HMG-CoA reductase in hepatic microsomal homogenates was no different than the activity determined for saline-injected controls (25,26). Yet, as determined in this study, the activity of HMG-CoA reductase was increased approximately by 3-fold 24 h after

the administration of P-407. It should be noted that we have previously determined that the activity of HMG-CoA reductase returns to normal levels at approximately 72 h following a single dose (0.5 g kg^{-1}) of P-407 to rats (27). Similar to the increase in enzymatic activity, we have also shown in the present study that there was a significant increase in the protein expression of HMG-CoA reductase in the liver 24 h after P-407 administration. This finding is consistent with the enormous increase in plasma cholesterol that we measured in P-407-treated mice. However, it should be noted that, unlike the trend observed with the HMG-CoA reductase protein expression data, the gene expression (mRNA levels) of HMG-CoA reductase was previously reported to be significantly reduced when P-407 was repeatedly administered (0.5 g kg^{-1} every 3 days by i.p. injection) for 200 days (25). Thus, it may be that sustained hypercholesterolemia induced by the continued administration of P-407 causes the gene expression of HMG-CoA reductase in mice to be down-regulated in an attempt to maintain cholesterol homeostasis, or sterol balance, across the animal without affecting the enzyme's activity.

Taken together, the down-regulation of the LDL receptor and the up-regulation of cholesterol biosynthesis create a scenario that favors the development of atherosclerotic lesions, in a similar way as the LDL receptor knockout/high cholesterol diet in the work of Isibashi *et al.* (2). Interestingly, the use of atorvastatin in the P-407 mouse model has been shown to induce the regression of atherosclerotic lesions (28). Millar *et al.* reported (4) the effect of P-407 in TG production after 2 h of drug treatment in control and LDLr knockout mice. At this early stage, there were no differences in the TG production rate.

It is worthwhile to compare and contrast our findings with those of other groups that used a structurally similar nonionic surfactant called Triton WR 1339. For example, at 6–9 h after intravenous injection of Triton WR 1339 in rats, Kuroda *et al.* (29) demonstrated that hepatic HMG-CoA reductase activity was increased 4- to 5-fold over baseline

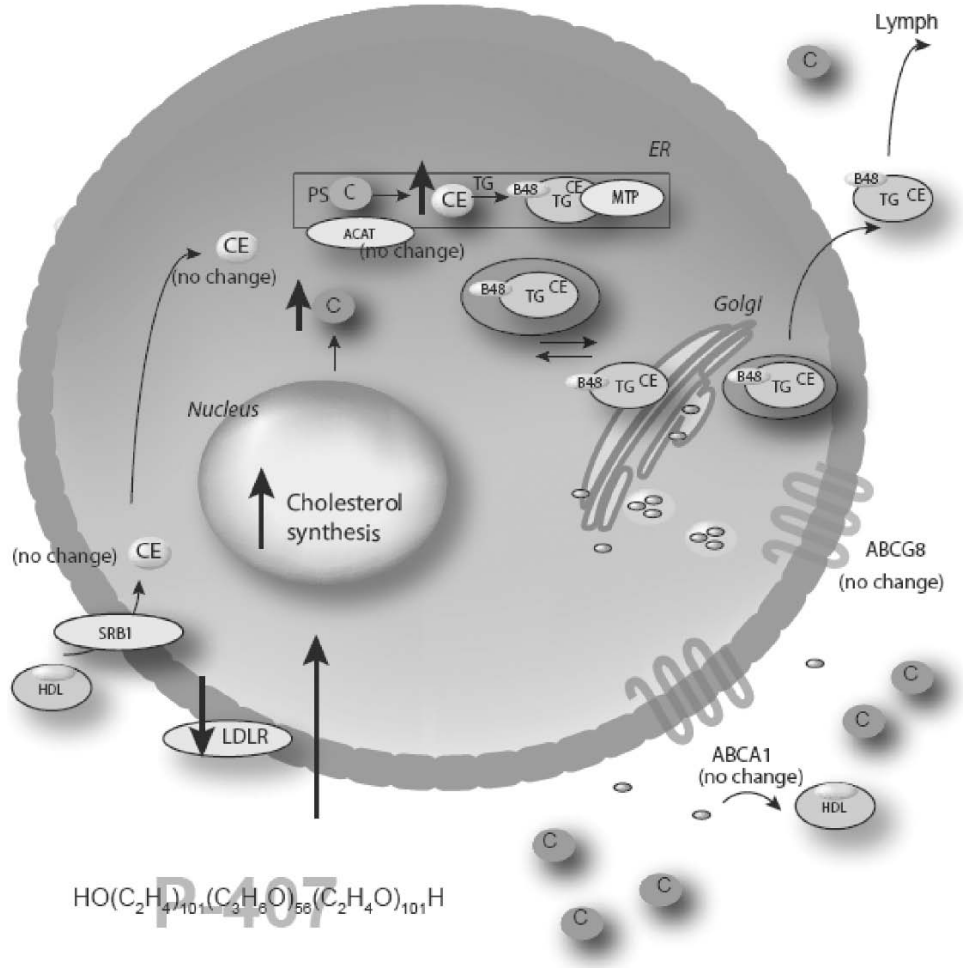

Fig. 7. Mechanisms of P-407 induced hypercholesterolemia in a mouse model. P-407 administration induced hepatic HMG-CoA reductase activity, whereas there was no effect on ACAT activity. SR-BI expression was not affected by P-407 treatment; however, the LDL receptor was significantly reduced. ABCA1 and ABCG8 expression was not different in control and P-407-treated liver extracts. In previous studies, we determined the inhibition of lipoprotein lipase (22) in this P-407 mouse model. We have also described the inhibition of microsomal cholesterol 7alpha-hydroxylase (C7alphaH) in a long-term P-407 treatment of mice (26).

activity. Anderson and Dietschy (30) showed that cholesterol synthesis significantly increased in six organs in addition to the liver after 3 days of Triton WR 1339 infusion to rats. The liver was suggested to be the organ primarily responsible for the newly synthesized cholesterol because of its relative mass and potential for cholesterolgenesis. This conclusion was also supported by the observation that hepatectomy, followed by Triton WR 1339 injection, did not cause an increase in the plasma cholesterol concentration (31). The chemical similarity of the nonionic surface-active agents Triton WR 1339 and P-407 caused by the polyoxyethylene ether bonds (32,33) may suggest a common mechanism(s) for induction of hypercholesterolemia by P-407.

Figure 7 summarizes our findings. P-407 administration to mice did not seem to alter either the biological activity or the expression of hepatic ACAT. SR-BI levels remained unchanged in liver extracts of P-407-treated mice, showing that changes in SR-BI expression may not be an explanation for higher liver CE levels in P-407-treated animals. However, the LDL receptor protein expression was significantly reduced in liver extracts from P-407-treated mice, suggesting that this may be a mechanism to explain decreased cholesterol internalization and subsequently higher cholesterol plasma levels. Twenty-four hours after P-407 administration, we did not detect a decrease in the protein expression of ABCA1 and ABCG8, which may have potentially accounted for the increased plasma cholesterol levels in these animals.

Block copolymers analogous to P-407, for example, Pluronic P85, have been shown to inhibit drug efflux transporters such as MRP1, MRP2, and P-glycoprotein (34,35). P85 inhibitory effect on P-glycoprotein ATPase activity is considerably greater compared to the effect on the other transporters. This is particularly interesting in that the role of P-glycoprotein on cholesterol homeostasis has been a matter of recent debate (36,37).

The activity and the protein expression of HMG-CoA reductase were increased in P-407-treated mice relative to controls. Thus, our data would seem to suggest that the increased levels of CE in the liver (observed in both mice and rats) and peripheral tissues (only rats evaluated to date) of rodents administered P-407 is not associated with an up-regulation of ACAT activity and/or protein expression of ACAT2, the major ACAT hepatic isoform. Rather, to maintain cellular cholesterol homeostasis and compensate for the expanded pool of cholesterol that result from an up-regulation of HMG-CoA reductase, our data would tend to support the conclusion that free cholesterol was converted to CE at a normal rate for subsequent storage as lipid droplets. Thus, acute P-407 administration to mice results in elevated liver CE concentrations by increasing hepatic cholesterol synthesis and decreased cholesterol internalization via the LDL receptor without altering hepatic cholesterol esterification and cellular efflux.

In conclusion, the increased hepatic CE levels, following P-407 administration, was neither related to an up-regulation of ACAT2, nor did it enhance SR-BI expression. Hypercholesterolemia was associated with an up-regulation of both the protein expression and activity of HMG-CoA reductase and decreased LDLr expression. Transporters other than ABCA1 and ABCG8 may be involved in this diet-independent murine model of hyperlipidemia.

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