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Poloxamer 407 (P-407)-mediated reduction in the gene expression of ATP-binding-cassette transporter A1 may contribute to increased cholesterol in peripheral tissues of P-407-treated rats

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Abstract

The purpose of this study was to determine whether poloxamer 407, a chemical known to increase plasma lipid levels in rodents following parenteral administration, decreased the gene expression of ATP-binding-cassette transporter A1. Using human macrophages cultured with poloxamer 407, there was a significant reduction in the gene expression of ATP-binding-cassette transporter A1; however, there was no effect on the gene expression of either fatty acid synthase or sterol regulatory element binding protein-1. Reduction of ATP-binding-cassette transporter A1 mRNA levels was also observed in both liver and intestine of poloxamer 407-treated rats. When macrophages were cultured with poloxamer 407, the percent of cholesterol effluxed decreased in a concentration-dependent fashion, both in the absence and presence of a synthetic liver X receptor agonist. Lastly, total and unesterified (free) cholesterol concentrations were determined in the liver and 9 peripheral tissues of poloxamer 407- and saline-injected (control) rats. In every tissue, the concentration of total cholesterol for poloxamer 407-treated rats was significantly greater than the corresponding value for controls. Our findings would seem to suggest that the poloxamer 407-mediated reduction in both ATP-binding-cassette transporter A1 gene expression and cellular cholesterol efflux may potentially be one factor that contributes to the accumulation of cholesterol and cholesteryl esters in the liver and 9 peripheral tissues of poloxamer 407-treated rats. Furthermore, the surprising specificity by poloxamer 407 for inhibition of ATP-binding-cassette transporter A1 gene expression over fatty acid synthase and sterol regulatory element binding protein-1 may potentially be due to either disruption of a transcriptional cofactor required for ATP-binding-cassette transporter A1 gene expression, or enhanced turnover of ATP-binding-cassette transporter A1 mRNA.

Keywords: Reverse cholesterol transport; Liver X receptor agonist; ATP-binding cassette transporter A1; Cellular cholesterol efflux

1. Introduction

While cholesterol is an essential component of mammalian cell membranes, excess cellular cholesterol is toxic and contributes to several diseases, most notably atherosclerosis. Previously, we have reported on a non-genetically-altered, non-diet-induced mouse model of atherosclerosis (Johnston, 2004). The animal model involves the parenteral administration of a compound called poloxamer 407 (P-407), a hydrophilic triblock copolymer comprised of polyoxy ethylene and polyoxypropylene units, to 5-week-old male or female C57BL/6 mice. A dose-dependent hyperlipidemia is observed as early as 1 h following P-407 administration and lasts approximately 5 days following a single dose. P-407 elicits the same dyslipidemic response in Sprague–Dawley rats. If chronic administration of P-407 to mice is maintained for 16 weeks, aortic atherosclerotic lesions are formed that are comparable in size and number to

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those observed using classic high-fat, diet-induced mouse models of atherogenesis (Johnston, 2004).

Understanding of cellular cholesterol homeostasis and its regulation has unfolded over several decades (Vance and Van den Bosch, 2000). As it pertains to coronary heart disease 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 which accumulate in cells when intracellular cholesterol concentrations are elevated are ligands for the liver X receptor nuclear receptors, which stimulate expression of ATP-binding cassette transporters (specifically A1, G1, G5/G8) and other genes involved in reverse cholesterol transport (Repa and Mangelsdorf, 2002). Several liver X receptor agonists are now being evaluated for their potential to increase the gene expression of ATP-binding-cassette transporter A1 which, in turn, increases the efflux of excess cellular cholesterol to lipid-poor, high-density lipoprotein for its eventual return to the liver. The antiatherogenic effect of liver X receptor agonists opposes the first step in atherosclerosis; namely, the formation of foam cells by cholesterol accumulation in arterial wall macrophages.

The purpose of the present study was to determine whether P-407 decreased the gene expression of ATP-binding-cassette transporter A1 in human macrophages. Our reason for wanting to determine whether the gene expression of this transporter was reduced in macrophages cultured with P-407 stemmed from our finding of increased total cholesterol and cholesteryl esters in homogenates of the liver and various peripheral tissues of P-407-treated rats. We hypothesized that the accumulation of cholesterol in homogenates of the liver and peripheral tissues of P-407-treated rats may have occurred for several reasons; including, but not limited to, an increased rate of cellular cholesterol synthesis, defective cholesterol export from cells (possibly due to P-407's effect on ATP-binding-cassette transporter A1), and/or redistribution of elevated plasma cholesterol to the cells of peripheral tissues for storage as cholesteryl esters. Since we had previously demonstrated (Johnston and Palmer, 1997) that a single injection of P-407 to rats temporarily increased (for approximately 35 h post-dosing) the hepatic microsomal activity of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, we now sought to determine whether P-407 modulated the gene expression of ATP-binding-cassette transporter A1 and, hence, the extent of cellular cholesterol efflux.

Using human macrophages cultured with increasing concentrations of P-407, we first demonstrated that the gene expression of ATP-binding-cassette transporter A1 was significantly decreased. Not surprisingly, the apolipoprotein AI-facilitated efflux of cholesterol from macrophages cultured with P-407 was also decreased. Cholesterol efflux from macrophages was also evaluated in both the absence and presence of a synthetic liver X receptor agonist to ascertain whether or not P-407 could potentially interact with and, subsequently, inhibit the drug's action. Because liver X receptor agonists can also affect the gene expression of sterol regulatory element binding protein-1 and fatty acid synthase (Edwards et al., 2002; Joseph et al., 2002), we also investigated whether P-407 had any effect(s) on the gene expression of both fatty acid synthase and sterol regulatory element binding protein-1 in cultured macrophages in both the absence and presence of the liver X receptor agonist. Lastly, to corroborate our findings in cell culture, we compared the levels of ATP-binding-cassette transporter A1 mRNA in both liver and intestinal tissue of P-407-treated and control rats.

2. Methods

2.1. Materials

Male Sprague–Dawley rats (200–225 g) were obtained from Charles River Laboratories (Wilmington, MA). P-407 was a gift from the BASF Corporation (Mount Olive, NJ). Assay kits for the measurement of total and free cholesterol were obtained from Wako Chemicals, Inc. (Richmond, VA). The P-407 solution for intraperitoneal (i.p.) injection was prepared by combining the agent with sterile water for injection U.S.P., and refrigerated overnight to facilitate dissolution of P-407 by the 'cold method' of incorporation (Schmolka, 1991). The liver X receptor agonist, GW683965A [hereafter abbreviated as GW965; $C_{33}H_{31}ClF_3NO_3{\cdot}HCl$ (Collins et al., 2002)], was provided by GlaxoSmithKline, Research Triangle Park, NC, USA. Leukopacks enriched for monocytes were obtained from Biological Specialty Corporation (Colmar, PA). Kits and equipment for the isolation of purified monocytes were obtained from Miltenyi Biotec (Auburn, CA). Acetylated low-density lipoprotein was obtained from Biomedical Technologies (Stoughton, MA); [^{3}H]-cholesterol was acquired from PerkinElmer Life and Analytical Sciences (Boston, MA). Apolipoprotein AI was obtained from Intracel Corp. (Frederick, MD). Primers and probes used for real-time polymerase chain reaction analysis in the human macrophage studies were as follows: sterol regulatory element binding protein-1: Assays-on-Demand catalog # Hs00231674_m1 (Applied Biosystems); ATP-binding-cassette transporter A1: left CAGTTCTGATGCTGGCCTGG, right TGCTTCCTGATGAGGTTGGAGA, probe 6FAM-CAGCGACCATGAGAGTGACACGCTGAC-TAMRA; fatty acid synthase: left ACCTGGGCGCGGACTAC, right CGATGACGTGGACGGATACTT, probe 6FAM-ACCTCTCCCAGGTATGCGACGGG-TAMRA; ribosomal protein L32 (RPL32): left CGCTCACAATGTTTCCTCCA, right TGACTCTGATGGCCAGTTGG, probe VIC-CGCAAAGCCATCGTGGAAAGAGCT-TAMRA.

The primers used for polymerase chain reaction analysis of ATP-binding-cassette transporter A1 gene expression in liver and intestine of P-407-treated and control rats were as follows; ATP-binding-cassette transporter A1: Forward 5′-ATCTCATAGTATGGAAGAATGTGAAGCT-3′, Reverse 5′-CGTACAACTATTGTATAACCATCTCCAAA-3′ (product size = 132 bp) (Hoekstra et al., 2003), glyceraldehyde-3-phosphate dehydrogenase: Forward 5′-TGAAGGTCGGAGTCAACGGAT-3′, Reverse 5′-TCGCTCCTGGAAGATGGTGAT-3′ (product size = 576 bp) (Takara et al., 2003).

2.2. P-407 administration

Rats 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 institution's guide for the care and use of laboratory animals, and the treatment protocol was approved by the Animal Care and Use Committee 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 of 10 rats were individually administered a 1.0 g/kg dose of P-407 (0.5 ml) by i.p. injection. 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 (Palmer et al., 1998). Thus, tissue samples were all harvested 24 h post-dosing. The second group of 10 rats were each administered 0.5 ml of sterile normal saline by i.p. injection and similarly treated.

2.3. Tissue collection

On the day of sacrifice (exactly 24 h after receiving either a dose of P-407 or normal saline) each animal was lightly anesthetized with ether and the peritoneal cavity opened, the descending abdominal aorta isolated, and a blood sample (6 ml) obtained. Blood was collected into heparinized (100 units/ml) glass 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 then centrifuged at 10,000×*g* for 10 min at 4 °C, the plasma harvested, and the plasma samples frozen at −80 °C until the time of analysis.

A small sample (100–200 mg) of the following tissues were immediately obtained postmortem; brain, lung, kidney, spleen, stomach, small intestine (duodenum), heart, muscle (gastrocnemius), retroperitoneal adipose tissue, and liver. 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 lipid analysis.

2.4. Lipid analysis

To confirm that the dose of P-407 injected into the peritoneal cavity was absorbed and elicited its hypercholesterolemic effect, frozen plasma samples were thawed, vortexed, and a 20 µl or 50 µl aliquot analyzed for total or free cholesterol, respectively, using a standard enzymatic, colorimetric assay kit. The assay is based on the technique described by Allain et al. (1974) 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 one standard deviation of the previously reported mean value of the C_{max}. All assays were performed in duplicate.

For analysis of total and free cholesterol in tissue specimens, samples were thawed, and then homogenized in a 3:1 ethanol/ether mixture as described by Entenman (1957). The samples were then evaporated to dryness at 90 °C. Residues were solubilized with 3 ml of 2-propanol and then assayed as described above for total and free cholesterol contained in plasma. The cholesterol standard provided with the kit had a concentration of 100 mg/ml 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 cholesteryl ester content of the harvested tissues was simply calculated as the difference between the total and free cholesterol concentrations. The concentrations of total cholesterol, free cholesterol, and cholesteryl ester in a given tissue were expressed in mg/g of tissue.

2.5. Isolation and culture of primary human macrophages

Human leukopacks were washed twice in Hank's balanced salt solution (without calcium) plus 1.0 mM EGTA and layered onto a 10 ml Histopaque gradient (Sigma). After centrifugation, the harvested interface layer was depleted of non-monocytes by incubation with a cocktail of hapten-modified monoclonal anti-Cluster of Differentiation (CD)3, CD7, CD19, CD45RA, CD56, and immunoglobulin E antibodies (Miltenyi Biotec, Inc., Auburn, CA) followed by capture of antibody-bound cells with anti-hapten antibody-coated magnetic cell sorting MicroBeads. Monocytes were collected, washed, and plated into T150 flasks in growth medium [Roswell Park Memorial Institute (RPMI)-1640 with 2 mM L-glutamine, 5% human, Type AB serum (Sigma), 100 U/ml penicillin and streptomycin, and 1 ng/ml granulocyte-monocyte colony stimulating factor (Invitrogen)]. Cells were allowed to differentiate in culture 7–10 days prior to cholesterol efflux and gene expression experiments, at which point they had reached nearly 100% confluence and purity.

2.6. ATP-binding-cassette transporter A1, fatty acid synthase, and sterol regulatory element binding protein-1 gene expression measurements in human macrophages

For gene expression measurement, 96-well plates were seeded with human macrophages in parallel with the cholesterol efflux assays. After adhering overnight, cells were treated for 24 h with dimethyl sulfoxide (DMSO), P-407, GW965, or combinations thereof, as indicated in the figure legends. For each treatment, 6 wells were treated identically. RNA was isolated according to the RNeasy 96 protocol for isolation of total RNA from animal cells (Qiagen). Briefly, cells were lysed in RLT Buffer (Qiagen) containing 1% β-mercaptoethanol. RNA was isolated using RNeasy 96 kits (Qiagen) involving vacuum technology. Following treatment with RNAse-free Dnase (Qiagen), RNA was eluted in 170 µl RNase-free water. RNA was quantitated with a TECAN GENios model spectrophotometer using the Ribogreen method according to the manufacturer's instructions (Molecular Probes).

Complimentary DNA (cDNA) was synthesized from total RNA with SuperScript™ II Reverse Transcriptase (RT) according to the manufacturer's instructions (Invitrogen). To approximately 50 ng RNA in 5 μl was added 2 μl (100 ng) random hexamers, 1 μl (10 nmol) dNTPs and 2 μl RNase-free water. Samples were heated at 65 °C for 10 min and then cooled on ice for 1 min. To this was added 2 μl of 10 × RT Buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M dithiothreitol, and 1 μl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). Following incubation at 25 °C for 2 min, 1 μl of SuperScript™ II RT (50 Units) was added. Plates were transferred to a PerkinElmer GeneAmp 9600 thermocycler and reactions were incubated at 25 °C for 10 min, followed by incubations at 42 °C for 50 min and 70 °C for 15 min. RNaseH (1 μl) was added and the reactions then incubated at 37 °C for 20 min. Finally, the cDNAs were diluted with 45 μl of RNase-free water.

Real-time polymerase chain reaction was performed in an ABI Prism 7700 Sequence Detector using 5 μl of each cDNA reaction. Reactions (25 μl) consisted of 1.25 μl of 20 × primer-probe mix in RNAse-free water, 12.5 μl of 2 × TaqMan mix, and 6.25 μl RNase-free water. After initial denaturation at 95 °C for 10 min, reactions were subjected to 40 cycles of polymerase chain reaction, each cycle consisting of 9 s at 95 °C, followed by 60 s at 60 °C. For each sample, gene expression of ATP-binding-cassette transporter A1, sterol regulatory element binding protein-1, and fatty acid synthase was normalized to that of RPL32 and data were calculated as fold expression relative to the average of the dimethyl sulfoxide treatment group.

2.7. Macrophage cholesterol efflux studies

Prior to assay, floating human macrophages were seeded in 96-well plates at 2×10^5 cells/ml, 200 μl/well (Packard/PerkinElmer), and allowed to adhere overnight. For lipid loading, [^{3}H]-cholesterol was slowly added to acetylated low-density lipoprotein and incubated at 37 °C for 30 min, followed by the addition of regular growth media. All wells received 50 μl of this mixture, containing 50 μg/ml acetylated low-density lipoprotein and 5 μCi/ml [^{3}H]-cholesterol. Cells were incubated for 24 h.

Wells were then aspirated, washed, and placed in 100 μl regular growth media without phenol red or serum, containing 1% fatty acid-free bovine serum albumin and desired drug treatments (dimethyl sulfoxide, P-407, GW965, or combinations thereof), as indicated in the figure legends, and incubated for 24 h. For each treatment, 6 wells were treated identically. The next day, wells were aspirated, washed, and placed in 100 μl regular growth media without phenol red or serum, containing 0.1% fatty acid-free bovine serum albumin and, when desired, 5 μg/ml apolipoprotein AI. Drug treatments were replenished and cells were incubated for 24 h. The concentration of P-407 never exceeded 10 μM in the macrophage cell culture experiments. The decision to use P-407 at a concentration of less than or equal to 10 μM was based on previous studies (Johnston et al., 1992, 2003). Using cell cultures, Johnston et al. (1992, 2003) demonstrated that the proliferation of peripheral blood lymphocytes and human umbilical vein endothelial cells were not affected at P-407 concentrations less than 80 and 40 μM, respectively. Additionally, P-407 has been used both in cell cultures and in vivo and has not mediated the lysis of myocytes (Johnston and Miller, 1985), erythrocytes (Johnston et al., 1992; Johnston and Miller, 1985; Wang and Johnston, 1995; Atkinson et al., 1988; Pec et al., 1992), and hepatocytes (Muller, 1991).

For quantitation of cellular cholesterol efflux, cellular debris was first removed from conditioned media by filtration through 96-well multiscreen vacuum manifolds (Millipore Corp.). Two 50 μl aliquots of the filtered media per well were transferred to separate opaque white 96-well plates (Packard). Cells were lysed in 50 μl 0.1 M NaOH with gentle shaking. All wells then received 200 μl Microscint-20 scintillant (Packard). After sealing the plates and shaking overnight, radioactivity was quantitated in a calibrated TopCount instrument. Percent cholesterol efflux was calculated by dividing cpm in conditioned media by total cpm (cpm in conditioned media + cpm in cell lysate).

2.8. ATP-binding-cassette transporter A1 expression in liver and intestine

Real-time polymerase chain reaction analysis for ATP-binding-cassette transporter A1 mRNA detection in liver and intestine of control and P-407-treated rats was conducted to complement our cell culture studies in which we determined ATP-binding-cassette transporter A1 expression in human macrophages incubated with varying concentrations of P-407 in the absence or presence of GW965. Total RNA from rat liver and rat intestines was isolated using an RNeasy Mini Kit (Qiagen; Pdt. No. 74104) according to the manufacturer's instructions. Briefly, a slice of either liver or intestinal tissue was weighed, 1.0 ml of lysate buffer added, and a Rotor-stator homogenizer used to disrupt and simultaneously homogenize the tissue specimen. The tissue lysate was centrifuged for 3 min at maximum speed and the supernatant carefully transferred into a new 2 ml tube. Ethanol (600 μl; 70%) was added to the cleared lysate and mixed by repetitive pipetting. Up to 700 μl of the sample was then applied to an RNeasy mini column placed in a 2 ml collection tube, the tube was centrifuged for 15 s at 10,000×*g*, and the flow-through discarded by using a pipette. Next, 700 μl of washing buffer was added, the tube was again centrifuged for 15 s at 10,000×*g*, and the flow-through discarded. A second washing step was performed by adding 500 μl of washing buffer 2 and the tube centrifuged an additional 15 s at 10,000*g*. The last step was repeated and the tube was then centrifuged for 2 min to dry the RNeasy silica-gel membrane. To elute the RNA, 50 μl of RNase-free water was added directly to the RNeasy silica-gel membrane, which was then subsequently centrifuged for 1 min at 10,000×*g*.

The concentration of RNA was determined by measuring the absorption at 260 nm (A_{260}) in a spectrophotometer. The ratio of the readings at 260 nm and 280 nm provided an estimate of the RNA purity. An A_{260}/A_{280} ratio between 1.8 and 2.0 was accepted as representative of essentially pure RNA. The yield of total RNA was about 20 μg per isolation. Purified mRNA was reverse transcribed into single-stranded cDNA. The amount of DNA was determined using the Oligreen Assay (Molecular Probes).

Table 1
Lipid concentrations in the liver and peripheral tissues of P-407- and saline-treated rats

Lipid (Grp.)	Brain	Lung	Kidney	Spleen	Stomach	Small Intestine	Heart	Muscle	Adipose tissue	Liver
TC (Cont.)[a]	16.4±1.2	4.8±0.4	3.8±0.3	3.2±0.1	3.5±0.2	2.2±0.1	1.7±0.1	0.9±0.03	0.8±0.05	2.4±0.1
TC (P-407)	19.5±1.4[b]	6.1±0.6[b]	4.5±0.2[b]	3.9±0.2[b]	4.1±0.2[b]	2.8±0.2[b]	2.0±0.1[b]	1.1±0.07[b]	1.1±0.2[b]	2.7±0.1[b]
FC (Cont.)[c]	16.0±1.7	4.3±0.4	2.5±0.3	2.4±0.2	2.5±0.3	1.7±0.2	0.9±0.1	0.6±0.1	0.6±0.1	1.5±0.2
FC (P-407)	18.1±2.6	4.8±0.6	2.9±0.2	2.7±0.3	2.8±0.1	2.0±0.2	1.4±0.2[b]	0.7±0.1	0.3±0.06[d]	1.0±0.2[d]
CE (Cont.)[e]	0.4±0.05	0.5±0.1	1.3±0.2	0.8±0.1	1.0±0.1	0.5±0.1	0.8±0.07	0.3±0.04	0.2±0.03	0.9±0.1
CE (P-407)	1.4±0.2[b]	1.3±0.2[b]	1.6±0.3	1.2±0.1[b]	1.3±0.2	0.8±0.1[b]	0.6±0.04[d]	0.4±0.07	0.8±0.1[b]	1.7±0.2[b]

[a] Total cholesterol (mg/g of tissue±S.D.).
[b] Indicates a significant ($P<0.05$) increase compared to control.
[c] Total cholesterol (mg/g of tissue±S.D.).
[d] Indicates a significant ($P<0.05$) increase compared to control.
[e] Total cholesterol (mg/g of tissue±S.D.).

Aliquots of cDNA were subjected to polymerase chain reaction for detection of the ATP-binding-cassette transporter A1 gene. Glyceraldehyde-3-phosphate dehydrogenase was the housekeeping gene and served as the internal control. All primers used for these analyses were synthesized by the Nucleic Acids Protein Services Unit at the University of British Columbia. The polymerase chain reactions were conducted using an MJC-Research PTC-200 thermocycler. The initial denaturation process was performed at 94 °C for 10 min, followed by a total of 40 cycles at 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and then completed at 72 °C for 10 min with a final temperature of 4 °C. The internal control (glyceraldehyde-3-phosphate dehydrogenase) was added to the same reaction at cycle 8 or 9. A sample from each polymerase chain reaction product was subjected to electrophoresis on a 1.5% agarose gel. The fluorescent bands were visualized under ultraviolet light (UV-Epi Chemi II) and quantified with UVP-labworks software. Data obtained in this fashion was normalized using the glyceraldehyde-3-phosphate dehydrogenase densitometric values.

2.9. Statistical analysis

Data are expressed as the mean value±S.D. of the mean. Data in Table 1 and Figs. 1, 5A, and 6 were analyzed using a 2-tailed Student's *t*-test. Data in Fig. 3A, B, and C were analyzed using a 1-way analysis-of-variance. Comparison of mean values between treatment (P-407) and control groups was considered statistically significant if $P<0.05$.

3. Results

3.1. Tissue lipid levels

Fig. 1 shows the plasma cholesterol concentrations in rats 24 h after i.p. administration of either P-407 or saline. For reference purposes, the change in the cholesterol lipoprotein distribution resulting from P-407 administration is shown in Fig. 2 (Johnston et al., 1999). Fig. 2 shows that treatment of mice with P-407 induces a shift in cholesterol from the high-density lipoprotein fraction, typically observed in normal mice and rats, to the intermediate-density lipoprotein/low-density lipoprotein and very-low-density lipoprotein fractions (Johnston et al., 1999).

Table 1 lists the concentrations of total and free cholesterol and, by subtraction, the concentration of cholesteryl esters in each of the tissues from P-407- and saline-treated rats. The concentration of total cholesterol in every tissue of P-407-treated rats was significantly ($P<0.05$) greater than the corresponding value for saline-injected controls (Table 1). Only heart tissue from P-407-treated rats contained a significantly ($P<0.05$) greater concentration of free cholesterol than

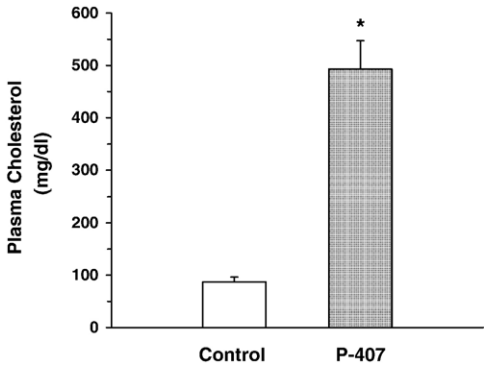

Fig. 1. Plasma total cholesterol concentrations 24 h after i.p. administration of either P-407 (1.0 g/kg) or saline to rats. All values represent the mean value± S.D. *Indicates a significant ($P<0.01$) increase compared to control.

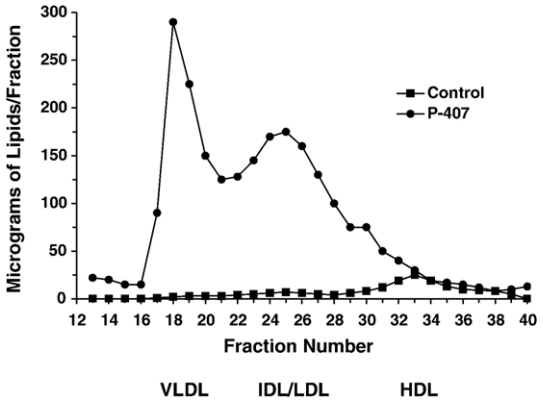

Fig. 2. The effect of P-407 (0.5 g/kg every 3 days) and normal saline (every 3 days) administration on the distribution of plasma cholesterol lipoprotein fractions. Reproduced from Johnston et al. (1999), with permission from Lippincott Williams & Wilkins, Inc., Philadelphia, PA.

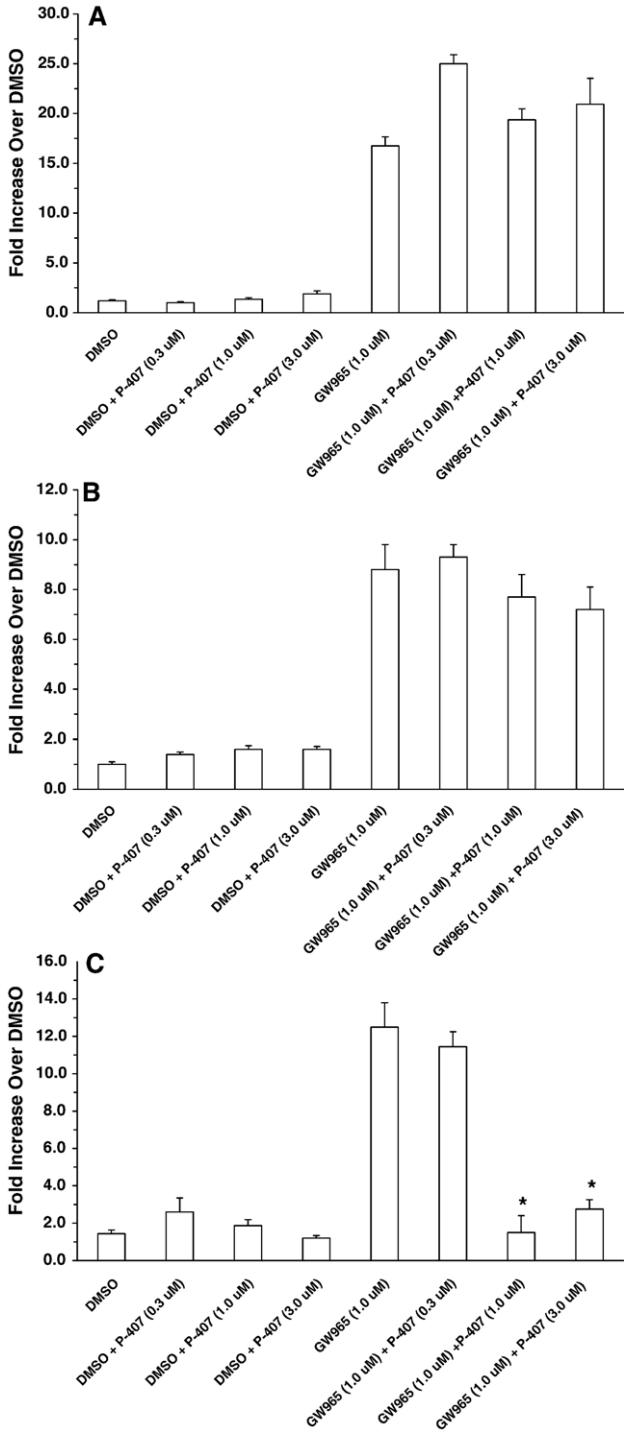

Fig. 3. (A) Fatty acid synthase expression in P-407-treated primary human macrophages in the presence or absence of GW965. All values represent the mean value±S.D. (B) Sterol regulatory element binding protein-1 expression in P-407-treated primary human macrophages in the presence or absence of GW965. All values represent the mean value±S.D. (C) ATP-binding-cassette transporter A1 expression in P-407-treated primary human macrophages in the presence or absence of GW965. All values represent the mean value±S.D. *Indicates a significant ($P<0.01$) decrease when compared to ATP-binding-cassette transporter A1 expression in 1.0 μM GW965 only.

the same tissue from saline-injected controls (Table 1). In fact, there was a significant ($P<0.05$) reduction in free cholesterol determined in both adipose tissue and liver in P-407-treated rats compared to these same tissues from controls (Table 1). With the exception of the heart, it can be noted that the calculated cholesteryl ester content of every tissue from P-407-treated animals was greater than the corresponding mean value for the same tissues obtained from controls. The increased cholesteryl ester content of tissues from P-407-treated rats reached statistical significance ($P<0.05$) in all tissues except kidney, stomach, and muscle. The cholesteryl ester content of heart from P-407-treated rats was significantly ($P<0.05$) less than the corresponding mean value calculated for saline controls (Table 1).

3.2. ATP-binding-cassette transporter A1, fatty acid synthase, and sterol regulatory element binding protein-1 gene expression in P-407-treated human macrophages

The effect of P-407 on expression of liver X receptor target genes ATP-binding-cassette transporter A1, fatty acid synthase, and sterol regulatory element binding protein-1 was examined both in control and liver X receptor-stimulated macrophages. It can be noted from Fig. 3A, B, and C that all three genes were strongly up-regulated by GW965. Neither the gene expression of fatty acid synthase nor sterol regulatory element binding protein-1 was affected ($P>0.05$) over the P-407 concentration range of 0.3 to 3.0 μM, whether in the absence or the presence (1.0 μM) of the liver X receptor agonist. In contrast to fatty acid synthase and sterol regulatory element binding protein-1, there was a significant ($P<0.01$) reduction in the expression of ATP-binding-cassette transporter A1 in P-407-treated macrophages at P-407 concentrations greater than or equal to 1.0 μM in the presence of the liver X receptor agonist (Fig. 3C).

3.3. Macrophage cholesterol efflux

Fig. 4 clearly demonstrates that as the concentration of P-407 was increased in the cell culture, the percent of cholesterol effluxed from human macrophages decreased in a concentration-dependent manner. A similar trend was observed when the cholesterol efflux from macrophages was evaluated in the presence of the liver X receptor agonist (1 μM) and increasing concentrations of P-407 (Fig. 4).

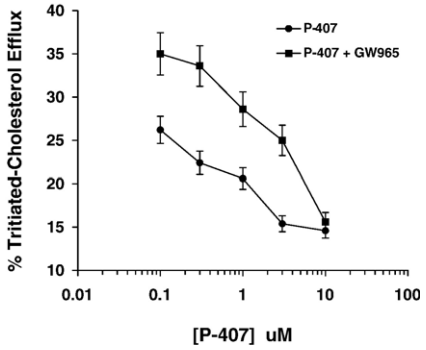

Fig. 4. Percent of apolipoprotein A1-mediated [^{3}H]-cholesterol efflux from human macrophages cultured with increasing concentrations of P-407 in either the absence or presence (1 μM) of the liver X receptor agonist, GW965. All values represent the mean value±S.D.

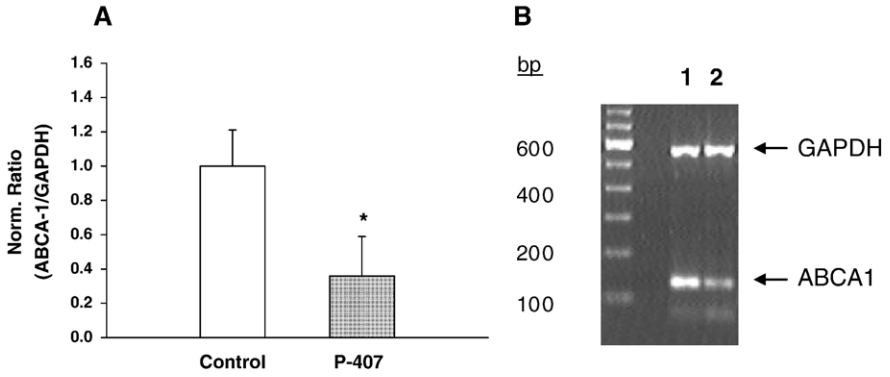

Fig. 5. (A) The effect of P-407 on ATP-binding-cassette transporter mRNA levels in liver obtained from P-407- and saline-treated (control) rats. All values represent the mean value±S.D. *Indicates a significant ($P<0.01$) decrease compared to control. (B) Representative agarose gel for ATP-binding-cassette transporter A1 expression in liver. Lanes 1 and 2 represent expression data for control and P-407-treated rats, respectively.

3.4. ATP-binding-cassette transporter A1 gene expression in liver and intestine

Fig. 5A and B clearly demonstrate down-regulation of ATP-binding-cassette transporter A1 gene expression in liver tissue of P-407-treated rats when compared to controls. The normalized ratio (ATP-binding-cassette transporter A1/glyceraldehyde-3-phosphate dehydrogenase) for liver tissue obtained from P-407-treated rats was significantly ($P<0.01$) less than the corresponding ratio for controls. This is confirmed by the markedly reduced intensity of the band for ATP-binding-cassette transporter A1 in lane 2 of Fig. 5B compared to the corresponding band for controls shown in lane 1. This same trend of reduced ATP-binding-cassette transporter A1 gene expression was also observed in intestinal tissue harvested from P-407-treated rats when compared to controls (Fig. 6). Similar to Fig. 5B, the corresponding agarose gel for ATP-binding-cassette transporter A1 expression in intestinal tissue demonstrated reduced band intensity relative to control (data not shown).

4. Discussion

Beginning with our in vitro studies, we have demonstrated that P-407 exerted a direct effect (reduced gene expression) on ATP-binding-cassette transporter A1 (a transcriptional target of liver X receptors) in human macrophages. Our results also demonstrated that fatty acid synthase and sterol regulatory element binding protein-1 expression were not affected when primary human macrophages were incubated in media with (1.0 μM) or without GW965 and P-407 over the concentration range of 0.3 to 3.0 μM. The fact that P-407 does not affect the expression of fatty acid synthase and sterol regulatory element binding protein-1 may suggest that P-407's effect is selective for ATP-binding-cassette transporter A1. Thus, it may be that P-407 disrupts interaction of liver X receptor with a cofactor required for transcription of the ATP-binding-cassette transporter A1 gene. Alternatively, P-407 may somehow dramatically increase ATP-binding-cassette transporter A1 mRNA turnover or catabolism.

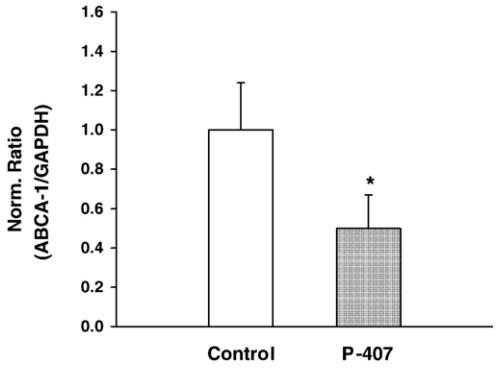

Fig. 6. The effect of P-407 on ATP-binding-cassette transporter A1 mRNA levels in intestine obtained from P-407- and saline-treated (control) rats. All values represent the mean value±S.D. *Indicates a significant ($P<0.05$) decrease compared to control.

Due to P-407's capacity to decrease the gene expression of ATP-binding-cassette transporter A1 in cultured macrophages, we next evaluated whether cholesterol efflux from cholesterol-loaded macrophages was impaired in the presence of P-407. We found that there was a concentration-dependent decrease in the percent of apolipoprotein A1-mediated cholesterol efflux from macrophages incubated with increasing concentrations of P-407 in both the absence and presence of the liver X receptor agonist. Not surprisingly, the curve representing macrophages cultured with P-407 plus 1 μM GW965 was positioned above the curve for P-407 only, since the liver X receptor agonist causes an increase in apolipoprotein A1-mediated cholesterol efflux from macrophages (Oram and Yokoyama, 1996; Oram, 2000).

Further support for our finding of reduced gene expression of ATP-binding-cassette transporter A1 in human macrophages cultured with P-407 was provided by the experiments which evaluated ATP-binding-cassette transporter A1 mRNA levels in liver and intestinal tissue obtained from P-407-treated rats. The results of those studies clearly demonstrated reduced gene expression of ATP-binding-cassette transporter A1 in the liver and intestine of P-407-treated rats relative to controls. In fact, the reduction in ATP-binding-cassette transporter A1 mRNA levels was more pronounced in liver compared to intestinal tissue.

The ATP-binding-cassette transporter A1 gene expression studies using macrophages and both liver and intestinal tissue

do not unequivocally demonstrate whether P-407-induced changes in this transporter's mRNA levels result from either a direct or indirect effect of P-407. Nevertheless, regardless of whether it resulted from either a direct or indirect process, the data contained in the present study would tend to support the conclusion that P-407 decreased the gene expression of ATP-binding-cassette transporter A1. Use of ATP-binding-cassette transporter A1 reporter gene constructs and nuclear run-on experiments are required to determine the degree to which the reduction in the mRNA levels may be due to effects at the level of transcription, RNA turnover, or both. In addition, our findings do not exclude the possibility that P-407 may directly or indirectly affect the activity of ATP-binding-cassette transporter A1 by reversible or irreversible alteration of the protein's structure and/or function. Lastly, it should be emphasized that a reduction in ATP-binding-cassette transporter A1 mRNA does not necessarily imply that the transporter's protein levels are similarly affected. However, recently, we have demonstrated that there is no difference in the protein expression of this transporter in the liver of P-407-treated rats relative to controls (Johnston, 2005, unpublished findings).

An additional piece of anecdotal evidence that supports P-407-mediated down-regulation of ATP-binding-cassette transporter A1 stems from a recent paper (Singaraja et al., 2002). Using mice, Singaraja et al. (2002) recently reported that increased ATP-binding-cassette transporter A1 activity protects against atherosclerosis. Since our in vitro findings demonstrated that P-407 caused both a down-regulation of ATP-binding-cassette transporter A1 gene expression, and, consequently, cellular cholesterol efflux, it would seem reasonable to suggest that such an effect might represent one of several factors that might contribute to the formation of aortic atherosclerotic lesions in mice treated with P-407 for 16 weeks. This would appear to be the predominant mechanism responsible for elevated cholesterol concentrations in the tissues following long-term treatment with P-407, since, unlike the temporary increase in the hepatic microsomal activity of 3-hydroxy-3-methylglutaryl CoA reductase observed following a single injection of P-407 (Johnston and Palmer, 1997), chronic administration of P-407 to mice for 16 weeks is not associated with a permanent increase in the activity of this enzyme relative to controls (Johnston et al., 2001). In contrast to extended, repetitive administration of P-407 for several months, a single, i.p. injection of P-407, as used in the present study, resulted in an increase in plasma cholesterol due to (a) a temporary and reversible redistribution of hepatic cholesterol to the vascular compartment (Johnston and Palmer, 1997), (b) an increase in the hepatic microsomal activity of 3-hydroxy-3-methylglutaryl CoA reductase for up to 35 h following P-407 administration, which exactly parallels the time course for depletion of hepatic cholesterol described in (a) (Johnston and Palmer, 1997), and (c) down-regulation in the activity of cholesterol 7α-hydroxylase (the rate-limiting enzyme involved with cholesterol elimination into bile), with subsequent accumulation of cholesterol in the plasma (Johnston et al., 2001).

It has long been known that excess cellular cholesterol is rapidly esterified with an unsaturated fatty acid to form cytoplasmic cholesteryl ester lipid droplets and that this reaction is catalyzed by the membrane-bound enzyme called acyl CoA:cholesterol acyltransferase (Chang et al., 2001). Our data demonstrated a statistically significant increase in cholesteryl ester levels in brain, lung, spleen, small intestine, adipose tissue, and liver from P-407-treated rats when compared to corresponding mean values in the same tissues of controls. Thus, perhaps the increased cholesterol levels in these tissues, which potentially results from both a P-407-mediated reduction in ATP-binding-cassette transporter A1 gene expression and its associated reduction in cellular cholesterol efflux, as well as an increase in cholesterol synthesis, causes an increase in the formation of cholesteryl esters to compensate for increased intracellular cholesterol stores. This may imply that P-407, either directly or indirectly, up-regulates the activity and expression of acyl CoA: cholesterol acyltransferase to facilitate the formation of cholesteryl esters. In fact, we previously reported increased activity for another acyltransferase; specifically, lecithin-cholesterol acyltransferase, in the plasma of P-407-treated rats when compared to plasma lecithin-cholesterol acyltransferase activity in control rats (Wasan et al., 2003). Presently, both in vitro and in vivo experiments are being performed to determine whether P-407 modulates the expression and/or the biological activity of acyl CoA:cholesterol acyltransferase; although our preliminary experiments to date would seem to suggest that neither the activity, nor the protein expression, of this enzyme is modulated following P-407 administration (Leon et al., 2005, unpublished findings).

Based on our present findings, this animal model may possibly prove useful for the study of Tangier disease, since it has now been proven that Tangier disease is caused by genetic mutations in ATP-binding-cassette transporter A1. This results in the accumulation of cholesterol and cholesteryl esters in macrophages located in the spleen, lamina propria of the intestine, tonsils, and in Kupffer cells of these patients (Attie et al., 2001). In addition, patients with Tangier disease normally exhibit a reduced plasma high-density lipoprotein concentration. It should be noted that we have previously reported that C57BL/6 mice treated with P-407 for 30 days exhibited a mean plasma high-density lipoprotein concentration of 35.1 ± 2.9 mg/dl, which was significantly ($P < 0.05$) less than the corresponding mean value for controls (54.2 ± 3.7 mg/dl) (Johnston et al., 1999). This may have potentially arisen from down-regulation in the gene expression of ATP-binding-cassette transporter A1, as well as a shift in the cholesterol lipoprotein distribution from high-density lipoprotein to predominantly the low-density and very-low-density lipoprotein fractions (Johnston et al., 1999); both effects being mediated by P-407.

In conclusion, we have shown that human macrophages incubated with increasing concentrations of P-407 in the presence of GW965 demonstrated reduced gene expression of ATP-binding-cassette transporter A1. In contrast, the gene expression of both fatty acid synthase and sterol regulatory

element binding protein-1 in similarly treated macrophages was unaffected. Our results demonstrating reduced ATP-binding-cassette transporter A1 mRNA levels in the liver and intestine of P-407-treated rats strongly supports our ATP-binding-cassette transporter A1 expression data obtained using human macrophages. Additionally, based on a concentration-dependent reduction in apolipoprotein A1-mediated cholesterol efflux from human macrophages cultured with P-407 in both the absence and presence of the liver X receptor agonist, we suggest that P-407 may be interfering with the role of ATP-binding-cassette transporter A1 in cholesterol export. Therefore, we propose that P-407 may potentially disrupt the interaction of liver X receptor with a cofactor required for transcription of the ATP-binding-cassette transporter A1 gene, or, alternatively, it may be that P-407 somehow dramatically increases ATP-binding-cassette transporter A1 mRNA turnover or catabolism. With either outcome, cellular cholesterol efflux would decrease, and may help to explain the significant increase in the concentration of cholesteryl esters we observed in the majority of the peripheral tissues harvested from P-407-treated rats. Ongoing work is aimed at unequivocally determining whether the expression and biological activity of acyl CoA:cholesterol acyltransferase is increased in P-407-treated rats to facilitate the esterification of excess cellular cholesterol to cholesteryl ester.

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