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The effect of poloxamer 407 on the functional properties of HDL in mice

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Abstract

Objectives There is an inverse relationship between high-density lipoprotein (HDL) and heart disease. HDL possesses not only both antioxidant and anti-inflammatory properties, but also anti-thrombotic and endothelial function-promoting qualities. However, it is not only the serum concentration of HDL that is important, but also the 'functional' quality of the HDL. The objective was to determine the functional status of HDL in a well-established mouse model of dyslipidaemia and atherosclerosis induced by the administration of a block copolymer (poloxamer 407; P-407).

Methods C57BL/6 mice were administered a single intraperitoneal dose of P-407 (0.5 g/kg) and blood was collected at 24 h post-dosing. HDL was isolated from controls (control HDL) and P-407-treated (P-407 HDL) mice and used to test its anti-inflammatory properties *in vitro*. Additionally, antioxidant enzymes associated with HDL, namely, platelet activating factor-acetylhydrolase (PAF-AH) and paraoxonase (PON), were evaluated for any potential reduction in their biological activity.

Key findings A single injection of P-407 in C57BL/6 mice resulted in a marked decrease in the levels of HDL-cholesterol and phospholipids. HDL particle size significantly increased, primarily due to remodelling of HDL with triglyceride. It was demonstrated that (i) long-chain saturated fatty acids were higher and the n-3/n-6 fatty acid ratio was significantly lower for P-407 HDL compared with control HDL, and (ii) P-407 HDL lost its capacity to inhibit tumour necrosis factor-α (TNF-α)-induced vascular cell adhesion molecule-1 (VCAM-1) expression compared with control HDL. Additionally, P-407 HDL was not able to neutralize lipopolysaccharide and inhibit subsequent TNF-α production compared with control HDL. The biological activity of platelet-activating factor acetylhydrolase (PAF-AH) and paraoxonase (PON) decreased in direct proportion to the circulating levels of both HDL-cholesterol and apolipoprotein (apoA-1).

Conclusions Combination of previously reported findings in P-407-treated mice, such as (i) production of both oxidized LDL and malondialdehyde, and (ii) profound elevations in the soluble forms of intercellular adhesion molecule-1 (ICAM-1), VCAM-1, and E-selectin, with the present results, would strongly suggest that HDL in P-407-treated mice is rendered dysfunctional. Thus, these findings help to explain why P-407-treated mice begin to form aortic atherosclerotic lesions about one month after initiating P-407 treatment.

Introduction

It is well known that there is an inverse relationship between high-density lipoprotein (HDL) and risk for cardiovascular disease in humans. The anti-atherogenic action of HDL is principally attributable to the reverse transport of cholesterol, whereby HDL promotes the efflux of cholesterol from peripheral cells to the liver. In addition, the HDL particle itself

has a variety of anti-inflammatory and antioxidative properties (e.g. HDL improves endothelial function by stimulating endothelial nitric oxide (NO) production). Also, it inhibits the expression of cell adhesion proteins such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin and, thereby, can inhibit inflammatory infiltrates accumulating in the vessel wall.[1] Specifically, HDL modulates the pro-atherogenic properties of any oxidized low-density lipoprotein (oxLDL) that may arise as a result of lipid peroxidation *in vivo* so that oxLDL cannot subsequently induce the increased expression of ICAM-1 and VCAM-1, as reflected by a rise in the plasma concentration of the shed, soluble forms (sICAM-1 and sVCAM-1).[2] Moreover, HDL can directly neutralize lipopolysaccharide (LPS) derived from Gram-negative bacteria, and inhibit the subsequent production of inflammatory cytokines.[3] However, it has been postulated that HDL particles may undergo chemical modifications in their protein or lipid components under conditions such as inflammation, diabetes and cardiovascular diseases. The HDL particle exhibiting these modifications may lose some, or all, of its atheroprotective effects, and induce inflammatory processes. HDL that has been transformed as such has often been referred to as 'dysfunctional' HDL.[1] These lines of evidence have stimulated tremendous interest in the functional quality and therapeutic potential of HDL in combating coronary artery disease (CAD).

HDL transports at least three enzymes, including lecithin-cholesterol acyltransferase (LCAT), paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH), which have putative protective roles in atherosclerosis. LCAT, which is secreted by the liver, plays a critical role in reverse cholesterol transport, a process whereby excess cholesterol is removed from macrophages-foam cells and esterified on HDL and returned to the liver for catabolism. Using a well-documented, rodent-based model of dyslipidaemia and atherosclerosis developed in our laboratory,[4–7] in which a copolymer (poloxamer 407; P-407) is administered by intraperitoneal injection to induce dyslipidaemia in either rats or mice of either sex, we have previously reported that the plasma LCAT activity in P-407-treated rats was four-fold to five-fold greater than that in saline-treated controls.[8]

PON, which is also synthesized and secreted by the liver,[8] is believed to inactivate phospholipid hydroperoxides formed during early events of lipoprotein oxidation.[9–11] Low plasma PON activity has been demonstrated in C57BL/6 mice maintained on an atherogenic high-fat, high-cholesterol, cholic acid-containing diet.[12] Moreover, PON-deficient C57BL/6 mice have increased aortic lesions compared with wild-type mice.[13] The deficiency in PON is also associated with an increase in HDL lipid peroxides. Data to date suggest that there is an inverse relationship between the biological activity of PON and atherosclerosis. Previously, we have demonstrated that P-407 was unable to oxidize native LDL *in vitro* as determined by measuring the rate of formation of conjugated dienes, as well as malondialdehyde (MDA) production using the thiobarbituric acid reactive substances (TBARS) assay.[14] In contrast, plasma obtained from C57BL/6 mice treated with P-407 for greater than two months demonstrated a significant ($P < 0.05$) increase in the content of oxidized lipids, as well as a significant ($P < 0.05$) elevation in both plasma IgG and IgM antibodies to MDA-modified LDL, or, in other words, oxLDL.[14]

The enzyme, platelet-activating factor acetylhydrolase (PAF-AH), secreted by macrophages, hydrolyses platelet-activating-factor (PAF) and other PAF-like lipids that are potent mediators of inflammation.[15] In humans, PAF-AH is associated primarily with LDL,[16] although a small fraction (15%) of the enzyme is found in the HDL density range. However, in mice, this enzyme is transported almost exclusively on HDL.[17,18] PAF and oxidatively-fragmented phospholipids appear to be substrates for the enzyme.[19] Watson *et al*.[11] have shown that this enzyme is able to hydrolyse oxidized phospholipids associated with minimally oxidized LDL, thus preventing monocyte binding to endothelial cells and the production of monocyte chemotactic protein-1 (MCP-1).

In this study, we sought to investigate the effect of P-407 on the functional status of HDL in the P-407-induced mouse model of dyslipidaemia and atherosclerosis. We determined whether the biological activity of both PON and PAF-AH were altered in P-407-treated mice in an attempt to identify additional factors that contribute to the formation of fibro-fatty aortic lesions in this mouse model of dyslipidaemia and atherosclerosis. This has important repercussions with regard to HDL's ability to prevent LDL oxidation and hydrolyse other oxidized phospholipids that play a pivotal role in the atherogenic cascade. Importantly, we characterized several functional properties of HDL isolated from P-407-treated mice (P-407 HDL), which are related to its anti-inflammatory effects. The overall goal was to determine whether this mouse model could be used to generate dysfunctional HDL *in vivo*. While dysfunctional HDL has been demonstrated in patients with CAD, the novelty of the present investigation resides in developing a convenient animal model that consistently reproduces the production of dysfunctional HDL in dyslipidaemic mice.

Materials and Methods

Materials

Fourteen male C57BL/6 mice, ~20 g, were purchased from the Jackson Laboratory (Bar Harbor, USA) and used for the experiments which assessed apoA-1, PAF-AH and PON. The remaining 90 C57BL/6 mice, ~20 g, were purchased from Crea Japan (Tokyo, Japan), and were utilized in the experi-

ments which evaluated the anti-inflammatory properties of HDL *in vitro*. Poloxamer 407 (P-407) was purchased from Invitrogen (Carlsbad, USA). The EnzChek paraoxonase assay kit to measure plasma PON activity was purchased from Invitrogen Corporation (Carlsbad, USA). The PAF-acetylhydrolase kit to measure plasma PAF-AH activity was purchased from Cayman Chemical Company (Ann Arbor, USA). The ELISA kits for mouse and human tumour necrosis factor alpha (TNF-α) were obtained from R&D Systems (Minneapolis, USA) and Biosource (Camarillo, USA), respectively. Human umbilical vein endothelial cells (HUVECs), THP-1, human monocyte/macrophage cell line and J774 mouse monocyte/macrophage cell line were purchased from the American Type Culture Collection (Manassas, USA). TNF-α was purchased from Biosource (Camarillo, USA). Lipopolysaccharide (LPS) from *Escherichia coli*, serotype 055 : B5, was obtained from Sigma-Aldrich (St Louis, USA) and was ion-exchange chromatography grade.

In-vivo experiments

All procedures for poloxamer 407 (P-407) administration and subsequent blood collection were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), and the institution's guide for the care and use of laboratory animals, and the treatment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Missouri-Kansas City and Kobe University School of Medicine.

Initial experiments were conducted to determine the biological activity of PON and PAF-AH in the plasma of mice administered P-407. In this subset of experiments, we used two groups of mice ($n = 7$ mice per group). Group 1 consisted of mice treated with normal saline, while Group 2 consisted of mice treated with P-407 (0.5 g/kg). Mice were administered 0.5 ml of either normal saline (Group 1), or P-407 dissolved in normal saline (Group 2), by intraperitoneal injection and a blood sample (600 µl) was subsequently obtained from each mouse by peri-orbital sampling at 24 h post-dosing. Previously, we have shown that plasma apoB-containing lipids (e.g. LDL) and triglycerides reach a maximum plasma concentration, while HDL-cholesterol reaches a minimum plasma concentration, 24 h after a single injection of P-407.[4] The mice were allowed free access to drinking water, but not food, throughout the 24-h experiment. All blood samples were collected into heparinized tubes, the plasma obtained and the samples stored at −80°C until the time of enzyme activity determinations.

The second set of experiments was conducted to evaluate the anti-inflammatory properties of HDL *in vitro* using HDL that had been isolated from mice administered P-407 (i.e. P-407 HDL). C57BL/6 mice were treated with a single injection of P-407 (0.5 g/kg) ($n = 60$) or saline (control) ($n = 30$). Twenty-four hours later, whole blood was obtained by cardiac puncture using a heparin-containing syringe, and plasma was isolated by centrifugation. Pooled plasma was collected from two or three mice and HDL fractions were isolated by ultracentrifugation for subsequent in-vitro evaluation of HDL quality.[20,21] HDL isolated from P-407-treated mice or saline-treated mice was referred to as P-407 HDL or control HDL, respectively. Additionally, plasma HDL size was analysed with an on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides by HPLC (LipoSEARCH) from Skylight Biotech, Inc. (Akita, Japan).[20,22] In brief, a 5-µl serum sample was injected into two connected columns (300 × 7.8 mm) of TSKgel LipopropakXL and eluted by TSKeluent Lp-1 (Tosoh, Tokyo, Japan). The effluent from the columns was continuously monitored at 550 nm after an online enzymatic reaction with the Determiner L TC kit (Kyowa Medex, Tokyo, Japan), and the cholesterol concentration in lipoproteins and their subclasses was calculated as described.[23] The conversion of elution time to particle diameter was performed using a column calibration curve, a plot of logarithm of the particle diameter of standard samples, latex beads (Magsphere Inc, Pasadena, USA) 25 and 37 nm in diameter, and a high-molecular-weight calibrator (Pharmacia-GE Healthcare, Piscataway, NJ, USA) containing thyroglobulin (17 nm), ferritin (12.2 nm), catalase (9.2 nm), albumin (7.1 nm) and ovalbumin (6.1 nm) against their elution times.

Determination of plasma lipids and enzyme activity

The collected plasma samples were analysed for cholesterol, triglyceride and phospholipid levels in each lipoprotein fraction using standard biochemical assays. The plasma activity of PON and PAF-AH was determined with commercial assay kits according to the manufacturer's instructions. The EnzChek PON assay is a highly sensitive, homogeneous fluorometric assay (excitation/emission maxima of 360 nm and 450 nm, respectively) for the organophosphate activity of PON, and is based on the hydrolysis of a fluorogenic organophosphate analogue. The 96-well plate was read using a model DTX 800/880 Multimode Detector (Beckman Coulter, Inc., Fullerton, USA). The assay was performed in duplicate for each sample. Intra-assay and inter-assay coefficients of variation for measured activity was <3.8% and <7.6%, respectively.

The principle of the PAF-AH assay is based on 2-thio PAF, which serves as a substrate for all PAF-AHs. Final activity of PAF-AH was normalized for mass. All results were expressed as nmol/h/ml of PAF-AH activity. The 96-well plate was read using the same instrument as described above for PON and the assay was performed in duplicate for each sample.

Intra-assay and inter-assay coefficients of variation for measured activity was <4.9% and <5.8%, respectively.

To determine whether P-407 interfered with the assays, increasing amounts of P-407 were included into the substrate solution supplied in each kit to obtain final P-407 concentrations of 0.1, 1.0, 10, 100, 500 and 1000 μM. These concentrations of P-407 were not arbitrarily selected, but instead represent the range of P-407 plasma concentrations previously observed when animals were injected with a 0.5 g/kg dose of P-407.[24] Using PON as an example, we added 10 μl of control mouse plasma to each of the six tubes to initiate the enzymatic reaction. The six P-407/substrate (organophosphate analogue)/PON solutions were assayed in duplicate. A similar P-407 assay interference experiment was also conducted for PAF-AH.

Ultracentrifugation analysis of mouse high-density lipoprotein

The HDL fraction in the second set of experiments (aimed at evaluating anti-inflammatory properties) was obtained from the pooled mouse plasma by ultracentrifugation as described elsewhere.[25] Briefly, the plasma density (ρ) was adjusted to 1.063 g/ml with solid KBr and the plasma was then centrifuged for 12 h at 541 000*g* (TLA100.3 rotor; Beckman Instruments, Fullerton, USA) at 4°C. The infranatants containing HDL were harvested, re-adjusted to $\rho = 1.21$ g/ml, and subsequently centrifuged for 12 h at 541 000*g* (TLA100.3 rotor; Beckman Instruments) at 4°C. The top fraction, collected by cutting the tubes, was dialysed against a solution of 0.15 mol/l NaCl and 0.3 mmol/l EDTA to obtain the HDL fraction corresponding to $\rho = 1.063$–1.21 g/ml. Cholesterol, triglyceride and phospholipid in the HDL fraction were measured using commercially available kits (Wako Pure Chemicals, Osaka, Japan). Protein was quantitated by Lowry, and the purity of HDL was confirmed by agarose gel electrophoresis stained with Fat Red from Sigma-Aldrich. Very-low-density lipoprotein (VLDL) was defined as the $\rho < 1.019$ g/ml fraction.

Immunoblot and histological analyses

HUVECs were cultured on a 6-well plate to form a confluent monolayer, and then incubated with 10 μg/ml TNF-α for 24 h in the presence or absence of HDL isolated from control or P-407-treated mice. Whole-cell lysates were harvested and subjected to Western blotting for VCAM-1 expression with the aid of anti-VCAM-1 (Santa Cruz Laboratory, Santa Cruz, USA). THP-1 cells (5.0×10^4 cells/200 μl) were incubated with 1 μg/ml LPS for 36 h in the presence or absence of HDL (50 μg) isolated from control or P-407-treated mice. Culture medium was collected and TNF-α levels were measured by ELISA.

Gas chromatography/mass spectrometry analysis of fatty-acid composition

The HDL fraction (100 μl) was mixed with 20 μl of 200 μg/ml heptadecanoic acid (C17:0) as the internal standard. Whole fatty acids of the mixture were extracted and methylated with a fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan), and purified with a fatty acid methyl ester purification kit (Nacalai Tesque) according to the manufacturer's protocol. Each of the derivatized samples (1 μl) was injected into a gas chromatography/mass spectrometry (GC/MS) instrument (GCMS-QP2010 Plus; Shimadzu, Kyoto, Japan) with a split mode (split ratio 1 : 5) and separated with a DB-5ms GC capillary column (30 m × 0.25 mm i.d. × 0.25 μm d.f.). The injection temperature was set at 280°C, the helium gas flow rate through the column was kept at 43.0 cm/s, and the column temperature was maintained at 40°C for 2 min isothermally and then raised by 6°C/min to 320°C. Ions were generated with 70 KeV electron impact (EI) ionization, and scans were recorded over the mass range of 35–500 *m/z*. Fatty acid methyl esters were identified with a GC/MS Metabolite Mass Spectral Database (Shimadzu). Each fatty acid methyl ester was quantitatively analyzed after calibration with the internal standard and the protein concentration of each HDL fraction.

Cholesterol efflux assay

Cholesterol efflux experiments were performed as previously described with slight modifications.[26] In brief, 2.0×10^5 THP-1 human macrophages were plated per well on 24-well plates and cultured at 37°C for 24 h in RPMI-1640 containing 10% fetal bovine serum (FBS). Next, THP-1 macrophages were labelled with [^{3}H]cholesterol (1.0 μCi/ml) and Cu^{2+}-oxidized LDL (25 μg protein/ml) in the media containing 0.2% bovine serum albumin (BSA) for 24 h. The cells were washed with phosphate-buffered saline (PBS) and incubated for 24 h in RPMI-1640 containing 0.2% BSA. The media were replaced with RPMI-1640 containing 0.2% BSA in the presence or absence of HDL (25 μg protein/ml) for 4 h. The percentage cholesterol efflux was calculated by dividing the media-derived radioactivity by the sum of the radioactivity in the media and the cells. HDL-specific cholesterol efflux was calculated by subtraction of the percent efflux of BSA-only media from that of the HDL-present media.

Lipopolysaccharide-neutralizing capacity

Mouse HDL (10 μg) was pre-incubated with 100 ng/ml of LPS at 37°C for 1 h, followed by analysis of the limulus amoebocyte lysate (LAL) activity of LPS using an LAL assay kit (QCL-1000; Lonza Inc., Williamsport, USA) as described previously.[27]

Data analysis

The average values of the plasma concentrations of each lipoprotein fraction for P-407-treated mice were individually compared with the corresponding average values for the control mice using the Student's *t*-test.[28] Similarly, the average values of the plasma PON and PAF-AH activity for P-407-treated mice were individually compared with the corresponding average values for each enzyme's activity in control mice using the Student's *t*-test.[28] $P < 0.05$ was deemed statistically significant. For the comparison of more than two mean values, a classic one-way analysis-of-variance was utilized to determine significant differences. The Method of Scheffé post-hoc test was used to identify mean differences[29] if a significant *F*-value was calculated (i.e. for any *P*-value < 0.05).

Results

Plasma lipoprotein concentrations

As can be noted in Table 1, a single injection of P-407 into C57BL/6 mice resulted in a marked increase in plasma cholesterol levels. VLDL-cholesterol and LDL-cholesterol were increased by approximately 100 fold, from 0.14 mmol/l to 11.2 mmol/l and by three fold from 0.33 mmol/l to 0.99 mmol/l, respectively. In contrast, HDL-cholesterol was decreased from 1.78 to 0.96 mmol/l. In addition to these changes in lipoprotein cholesterol, P-407 treatment markedly increased the content of triglycerides in each lipoprotein fraction. In particular, the plasma concentration of VLDL-triglyceride was the lipoprotein fraction that demonstrated the greatest increase (200 fold) in response to P-407. Phospholipids were predominantly distributed in the HDL fraction at baseline. However, the phospholipid content in LDL and VLDL was markedly increased by P-407, while that of HDL was significantly decreased (Table 1). When HDL particle size was measured using HPLC, we found that the HDL particle size increased from 11.2 ± 0.02 nm to 11.7 ± 0.03 nm ($P < 0.01$). Thus, P-407 administration to mice resulted in an increased HDL particle size, as well as an HDL particle that was enriched with triglyceride. When we measured the plasma concentration of an HDL-associated apolipoprotein (apoA-1), it was significantly decreased (30%) by P-407 administration (Table 2).

Table 1 Fasting plasma lipoprotein concentrations in C57BL/6 mice before and 24 h after P-407 administration

	–P-407	+P-407	*P*-value
Cholesterol (mmol/l)			
HDL-CHOL	1.78 ± 0.06	0.96 ± 0.02	<0.01
LDL-CHOL	0.33 ± 0.03	0.99 ± 0.07	<0.001
VLDL-CHOL	0.14 ± 0.01	11.2 ± 0.23	<0.001
Triglyceride (mmol/l)			
HDL-TG	0.03 ± 0.01	0.25 ± 0.03	<0.001
LDL-TG	0.10 ± 0.02	1.40 ± 0.06	<0.001
VLDL-TG	0.32 ± 0.02	69.2 ± 2.4	<0.001
Phospholipid (mmol/l)			
HDL-PL	2.5 ± 0.11	1.1 ± 0.05	<0.01
LDL-PL	0.19 ± 0.03	0.69 ± 0.08	<0.01
VLDL-PL	0.28 ± 0.06	13.3 ± 0.30	<0.001

The high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) fractions were obtained by ultracentrifugation from 1.5 ml of pooled plasma from two or three male mice. The cholesterol (CHOL), triglyceride (TG) and phospholipid (PL) levels in each lipoprotein fraction were determined by using commercially available biochemical assay kits. Values are expressed as the mean ± SEM (mmol/l, n = 6–8 mice in each group).

Activity of circulating paraoxonase and platelet-activating factor–acetylhydrolase

We further evaluated the effect of P-407 on the biological activity of two HDL-associated enzymes. As noted in Table 2, the plasma activity of PON was significantly less ($P < 0.05$) in mice treated with P-407 (36.1 ± 4.2 Units/μl) compared with mice treated with saline (52.3 ± 5.8 Units/μl). Similarly, the plasma activity of PAF-AH was significantly less ($P < 0.05$) in mice treated with P-407 (2750 ± 185 nmol/h/ml) compared with mice treated with saline (3850 ± 251 nmol/h/ml) (Table 2). The reductions in the activity of PON and PAF-AH correspond to approximately a 31% and 29% decrease in enzyme activity, respectively, in P-407-treated mice compared with saline-treated controls. P-407, at incrementally increasing concentrations, neither interfered with the assay nor caused a reduction in the biological activity of either enzyme when pure substrate (supplied by the manufacturer) was used to assess the enzymatic activity of PON and PAF-AH in the presence of P-407.

Analysis of the fatty acid composition of the isolated high-density lipoprotein fraction

To expand our understanding of the characteristics of the lipid profile observed in P-407-treated mice, we assessed the fatty acid composition of the whole HDL fraction by means of gas chromatography-mass spectrometry (GC/MS). The high-throughput analysis revealed that P-407 treatment substantially altered the composition of fatty acids in HDL particles (Figure 1). Fatty acids have been shown to modulate inflammatory processes, with saturated fatty acids (SFA) directly stimulating TLR2-receptor or TLR4-receptor signalling, and n-3 polyunsaturated fatty acids (PUFAs) exerting a range of anti-inflammatory actions. At the same time, n-6 PUFAs were identified as a source of prothrombotic and pro-inflammatory eicosanoids. Interestingly, the content of long-chain saturated fatty acids was increased in P-407 HDL compared with control HDL (Figure 1). As a result, P-407 HDL was

Table 2 Changes in high-density lipoprotein (HDL)-associated proteins following induction of atherogenic dyslipidaemia with P-407

	Control mice	P-407-treated mice	Percent reduction	*P*-value
ApoA-1 (mg/dl)	82.0 ± 4.7	57.4 ± 3.2[a]	30.0	<0.05
PON (units/μl)	52.3 ± 5.8	36.1 ± 4.2[a]	30.9	<0.05
PAF-AH (nmol/h/ml)	3850 ± 251	2750 ± 185[a]	28.6	<0.05

C57BL/6 mice were administered P-407 (0.5 g/kg) by intraperitoneal injection. Twenty-four hours later, the plasma was obtained and the levels of apolipoprotein (apoA-1), as well as the biological activity of platelet activating factor-acetylhydrolase (PAF-AH) and paraoxonase (PON), were determined as described in the Methods. The percent reduction in PON and PAF-AH activity are very close to the percent reduction observed in the concentration of circulating apoA-1. [a]$P < 0.05$ compared with the mean value for controls (Student's *t*-test).

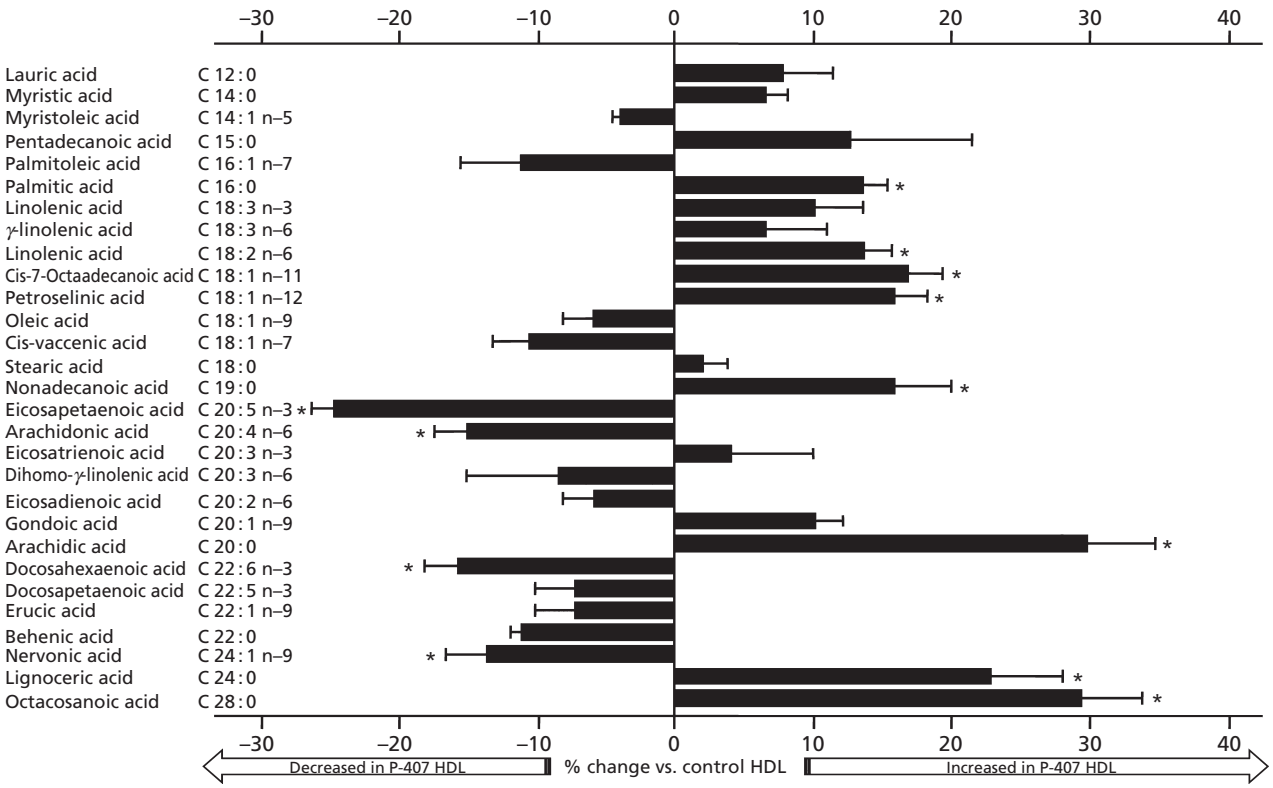

Figure 1 Analysis of the fatty acid composition of P-407 HDL by gas chromatography-mass spectrometry. C57BL/6 mice were treated with a single injection of P-407 or saline (control). Twenty-four hours later, pooled plasma was collected from two or three mice and the high-density lipoprotein (HDL) fraction was isolated by ultracentrifugation. The fatty acid composition of the HDL was evaluated by means of gas chromatography-mass spectrometry (GC/MS). P-407 HDL was significantly enriched in long-chain saturated fatty acids (SFA) when compared with control HDL. The n-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA), were decreased in P-407 HDL relative to control HDL. *$P < 0.05$ vs control HDL ($n = 5$).

significantly higher in SFA content than that observed in control HDL (~42% increase in the SFA/PUFA ratio relative to the SFA/PUFA ratio for control HDL; $P < 0.05$). Moreover, the n-3/n-6 fatty acid ratio was significantly lower for P-407 HDL than the corresponding ratio observed for control HDL (~21% decrease in n-3/n-6 ratio relative to the n-3/n-6 ratio for control HDL; $P < 0.05$). Considering that P-407-treatment changed the triglyceride content most robustly, we speculate that the change in the fatty acid composition after P-407-treatment most likely represents that in HDL triglycerides. These results would seem to imply that P-407 HDL may contain pro-inflammatory fatty acids compared with control HDL.

P-407 high-density lipoprotein demonstrates a loss in its atheroprotective properties *in vitro*

HDL has been shown to inhibit the expression of endothelial adhesion molecules induced by pro-inflammatory cytok-

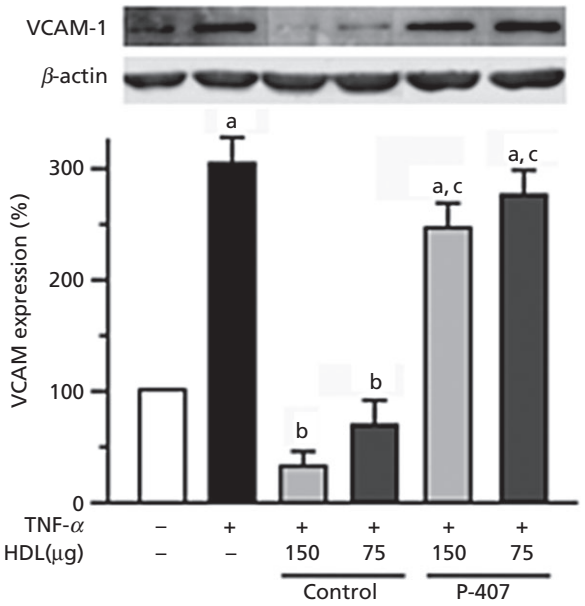

Figure 2 Effect of P-407 high-density lipoprotein (HDL) on tumour necrosis factor-α (TNF-α)-induced vascular cell adhesion molecule-1 (VCAM-1) expression in cultured human umbilical vein endothelial cells (HUVECs). HUVECs were cultured to form a confluent monolayer, and then incubated with 10 ug/ml TNF-α for 24 h in the presence or absence of HDL isolated from control or P-407-treated mice. Whole-cell lysates were harvested and subjected to Western blotting for VCAM-1 expression. $^{a}P < 0.01$ vs control (i.e. no TNF-α or HDL). $^{b}P < 0.01$ vs TNF-α only. $^{c}P < 0.05$ vs the corresponding equal amount of control HDL.

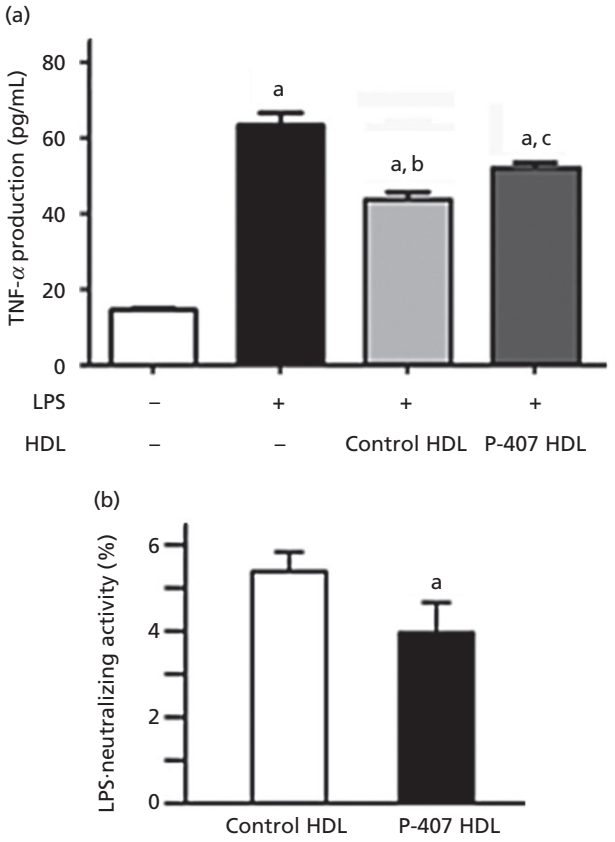

Figure 3 Effect of high-density lipoprotein (HDL) on lipopolysaccharide (LPS)-induced tumour necrosis factor-α (TNF-α) production in THP-1 macrophages. (a) THP-1 cells (5.0×10^4/200 μl) were incubated with 1 μg/ml LPS for 36 h in the presence or absence of HDL (50 μg) isolated from control or P-407-treated mice. Culture medium was collected and the TNF-α level was measured by enzyme-linked immunosorbent assay (ELISA). $^{a}P < 0.0001$ vs baseline (i.e. no LPS or HDL). $^{b}P < 0.01$ vs LPS only ($n = 10$). $^{c}P < 0.05$ vs LPS + control HDL ($n = 10$). (b) Mouse HDL (10 μg protein) obtained from P-407-treated and control mice was incubated with 100 ng/ml of LPS at 37°C for 1 h, and LPS-neutralization was quantified by the limulus amoebocyte lysate (LAL) assay. $^{a}P < 0.05$ vs control HDL ($n = 3$).

ines.[30] Because our HDL particle analysis suggested that the composition of HDL may be modulated by P-407 treatment, we investigated the effect of HDL on TNF-α-induced VCAM-1 expression in endothelial cells. As shown in Figure 2, TNF-α-induced VCAM-1 expression was significantly reduced by control HDL in a dose-dependent manner. However, when the same amount of HDL (protein) isolated from P-407-treated mice was evaluated, this inhibitory effect was abolished (Figure 2).

It is well known that HDL can form a complex with LPS,[31] which results in neutralization of LPS activity and attenuation of the subsequent release of inflammatory cytokines, including TNF-α. We therefore evaluated the effect of HDL on LPS-induced TNF-α production in THP-1 cells. Pre-incubation of LPS with control HDL resulted in a significant decrease in TNF-α production, while this inhibitory effect was attenuated when we utilized P-407 HDL (Figure 3a). Moreover, we compared the LPS-neutralizing capacity of HDL by using the limulus amoebocyte lysate assay. Our results demonstrated that this capacity was significantly ($P < 0.05$) lower using P-407 HDL than when we evaluated control HDL in the same assay (Figure 3b).

Cellular cholesterol efflux to high-density lipoprotein

The anti-atherogenic function of HDL is principally mediated by its ability to take cholesterol from peripheral cells and transport it back to the liver. Therefore, we finally assessed the promotion of cholesterol efflux by control HDL and P-407 HDL. As shown in Figure 4, the percent of cholesterol effluxed from macrophages to P-407 HDL was significantly ($P < 0.01$) less than the percent of cholesterol exported to control HDL (Figure 4). These findings would

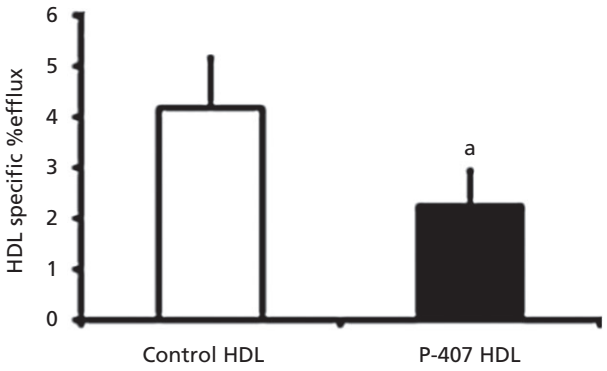

Figure 4 Effect of P-407 high-density lipoprotein (HDL) on cholesterol efflux from cholesterol-loaded macrophages. THP-1 macrophages loaded with [^{3}H]cholesterol were incubated with equal amounts of protein (25 μg/ml HDL) (a well-documented acceptor of cholesterol) from either saline-treated, or P-407-treated, mice as described in the Methods. Data are expressed as the percent of fractional efflux to the acceptor (either control HDL or P-407 HDL) during 4 h. Data represent three independent experiments with duplicate samples. Data are expressed as the mean ± S.D. $^aP < 0.01$ vs control HDL.

seem to suggest that P-407 HDL is significantly less effective in facilitating reverse cholesterol transport than control HDL.

Discussion

This study was undertaken to investigate whether dysfunctional HDL could be intentionally produced in P-407-treated mice. We have demonstrated that treating C57BL/6 mice with P-407 results in not only an atherogenic plasma lipid profile or phenotype, but also a loss in the anti-inflammatory properties of HDL. Importantly, we have also shown that two HDL-associated enzymes capable of providing antioxidant effects have undergone a significant and similar reduction in their biological activity. In demonstrating that HDL is dysfunctional in this model, we have identified yet another potential mechanism that contributes to our understanding of why mice treated with P-407 form aortic atherosclerotic lesions beginning about one month after initiation of treatment.

In terms of antioxidant properties of HDL, we have previously shown that P-407 does not directly oxidize LDL *in vitro*.[14] The 31% and 29% decrease in PON and PAF-AH activity, respectively, observed in the present study, parallels the 30% reduction of apoA-1. These findings would seem to imply that the decrease in PON and PAF-AH activity (~30%) may potentially mirror the decrease in plasma levels of HDL in P-407-treated mice, as suggested by the 30% reduction in the primary protein associated with HDL (i.e. apoA-1). As an aside, it is interesting to note that transgenic expression of apoA-1 increases HDL and delays atherosclerosis,[32–34] a trend that is just opposite to what we observed in P-407-treated mice (i.e. a decrease in apoA-1 and HDL and accelerated atherosclerosis). In view of the decrease in plasma apoA-1 we observed in this investigation and previously,[35] future experiments will be directed at determining whether there has been substitution of serum amyloid A (SAA) for apoA-1 in HDL (i.e. HDL that has been enriched with SAA).[36]

One of HDL's anti-atherogenic effects has been postulated to result from protection of LDL from oxidative modification, possibly mediated by HDL-associated PON and PAF-AH.[37] However, this would appear unlikely in P-407-treated mice, since we have previously reported that oxLDL and malondialdehyde are both present in the plasma of P-407-treated mice.[14] Based on the P-407 interference studies with the PAF-AH and PON assay kits, we would suggest, at a minimum, that P-407 does not directly interfere with the biochemical reaction between substrate and either enzyme. Therefore, by as yet some unknown mechanism, HDL in P-407-treated mice is not able to halt the formation of oxLDL and MDA.

Oxidation of the phospholipid content of LDL generates compounds with PAF-like bioactivity.[37] These substances induce the expression of cellular adhesion molecules (CAMs) such as VCAM-1 and ICAM-1 on the endothelial cell surface, and VCAM-1 and ICAM-1 expression can be prevented *in vitro* by antioxidants,[38] HDL and PAF-AH.[39,40] The HDL from P-407-treated mice is not only unable to stop the oxidative modification of LDL to form oxLDL, but also inhibit oxLDL from inducing the increased expression of CAMs, since we recently reported that plasma concentrations of sVCAM-1, sICAM-1, and sE-selectin all significantly increase within 48 h after a single injection of P-407 in mice.[41] This fact is corroborated in the present study by demonstrating that the inhibitory effect of HDL on VCAM-1 expression *in vitro* was attenuated by P-407 HDL compared with control HDL. Thus, in terms of an anti-inflammatory effect, P-407 HDL would appear to be 'dysfunctional'.

Although somewhat speculative in nature, it may be that the altered HDL lipid composition (i.e. HDL enriched with triglyceride and SFA), in addition to low phospholipids and n-3 PUFAs, plays a potential role in the reduced anti-inflammatory properties we observed with P-407 HDL. Loss of anti-inflammatory properties by triglyceride-enriched HDL obtained from P-407-treated mice would appear logical based on the findings of Patel *et al*.[42] Patel *et al*. infused 15 mg/kg/h Intralipid 20% fat emulsion or saline into eight fasting healthy human males and then isolated their HDL to determine its ability to inhibit VCAM-1 and ICAM-1 expression in human coronary artery endothelial cells stimulated with TNF.[42] The investigators found that the infusion of Intralipid resulted in significantly ($P < 0.05$) increased

triglyceride-rich HDL that exhibited impaired anti-inflammatory activity compared with HDL isolated from controls.

It is noteworthy to compare the activity of PON in our mouse model of dyslipidaemia and atherosclerosis with the activity of these enzymes in other mouse models of atherosclerosis. For example, Forte *et al*.[43] observed that apolipoprotein E-deficient mice, when fed a short-term (14 days) Western diet, had a 38% decrease in the activity of PON. However, these same authors noted that LDL-receptor-deficient mice, when placed on the same Western diet for 14 days, did not exhibit a reduction in the activity of PON.[43] Our data (~31% loss in the biological activity of PON) is in close agreement to that of Forte *et al*.[43]

Interestingly, low plasma PON activity has been demonstrated in C57BL/6 mice maintained on an atherogenic high-fat, high-cholesterol, cholic acid diet,[44] and it has been shown that PON-deficient C57BL/6 mice have increased aortic lesions compared with wild-type mice.[45] Studies with PON-apolipoprotein-E double knockout mice suggest that PON deficiency promotes LDL oxidation and atherosclerosis.[38] Since PON deficiency promotes LDL oxidation and atherosclerosis,[38] perhaps the 31% reduction in the biological activity of PON in P-407-treated mice contributes to the formation of oxLDL and eventual atherosclerosis.

The enzyme PAF-AH, which is secreted by macrophages, hydrolyses PAF and other PAF-like lipids that are potent mediators of inflammation.[40] In humans PAF-AH is primarily associated with LDL but in mice this enzyme is transported almost exclusively on HDL.[46,47] It is worth mentioning that P-407 itself might be suspected of acting as a potent mediator of inflammation. However, this would appear unlikely based on prior work from our laboratory. Previously, we investigated the direct effects of P-407 on endothelial cells and macrophage functions *in vitro*.[48] Our results demonstrated that incubation of P-407 with HUVECs in culture did not influence either cell proliferation or interleukin-6 and interleukin-8 production.[48] Thus, it was concluded that the formation of atherosclerotic lesions in our mouse model of atherosclerosis does not result from either direct stimulation of endothelial cells or macrophage activation by P-407.[48] Returning to the findings of Forte *et al*., these investigators demonstrated a significant reduction (~35%) in plasma PAF-AH activity when wild-type (control), apoE$^{-/-}$, and LDLr$^{-/-}$ mice were fed a Western diet compared with each genotype fed a standard chow diet.[43] Future research will include measurement of plasma concentrations of oxidized phospholipids (e.g. 1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC)) in P-407-treated mice, since Forte *et al*., observed a profound elevation in plasma POVPC and PGPC when wild-type, apoE$^{-/-}$, and LDLr$^{-/-}$ mice were fed a Western diet compared with each genotype fed a standard chow diet.[43]

The present investigation also demonstrated that LPS neutralizing capacity was less potent with P-407 HDL compared with control HDL. Previous studies have demonstrated that lipid-A binds to the phospholipids on the surface of HDL.[3] In fact, the present study showed that the phospholipid content in HDL particles was lower in P-407 HDL than in control HDL. Perhaps the phospholipids associated with P-407 HDL are released into the circulation and undergo oxidative modification. As mentioned above, this would further substantiate our desire to measure plasma concentrations of oxidized phospholipids (e.g. POVPC and PGPC) in future research. We would also suggest that the decreased phospholipid content of P-407 HDL may decrease the affinity of HDL particles for lipid-A, thereby causing a reduction in HDL's capacity to neutralize the biological activity of LPS. Furthermore, P-407 HDL was deficient in n-3 PUFAs (including EPA) and enriched in SFA, compared with control HDL. This could conceivably cause the HDL particles obtained from P-407-treated mice to be more pro-inflammatory than HDL particles isolated from control mice. The precise mechanism underlying the altered fatty acid composition remains equivocal and needs to be clarified with future additional studies.

As for the HDL-mediated cholesterol efflux, P-407 HDL exhibited significantly decreased cholesterol efflux compared with control HDL. The mechanism for the dysfunction remains unclear, but we would suggest that P-407 HDL may not be able to accept and bind cholesterol in the HDL particles because of increased triglyceride content. This finding suggests that reverse cholesterol transport is impaired by P-407 treatment, which may account for the hypercholesterolaemia and accelerated atherosclerosis observed in this particular mouse model of atherogenesis. Future experiments will also assess cholesterol efflux from cultured macrophages to serum, because Khera *et al*.[49] demonstrated that the efflux capacity of serum HDL was a strong predictor of CAD status, and that this association was independent of circulating HDL and apoA-1 levels.

Conclusions

In summary, the present findings suggest that HDL in P-407-treated mice is rendered dysfunctional, but not as a result of the direct action of P-407 on HDL. Thus, P-407-treated mice would appear to be a useful animal model with which to purposely generate dysfunctional HDL. Production of dysfunctional HDL may contribute, in part, to the formation of aortic atherosclerotic lesions observed in this mouse model of dyslipidaemia.

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